ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 5, pp. 636–642. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © D.V. Vorob'ev, N.P. Meichik, E.S. Lobakova, I.P. Ermakova, N.P. Matveeva, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 5, pp. 702–708.

EXPERIMENTAL ARTICLES

Ion-Exchange Characteristics of the Cell Walls Isolated from the Thallus of the Lichen *Peltigera aphthosa* (L.) Willd

D. V. Vorob'ev[1], N. P. Meichik, E. S. Lobakova, I. P. Ermakova, and N. P. Matveeva

Faculty of Biology, Moscow State University, Russia

Received November 24, 2008

Abstract—Ion-exchange characteristics of the cell walls isolated from different zones of the foliose lichen *Peltigera aphthosa* (L.) Willd were determined. Four types of ionogenic groups were revealed in the thallus cell walls of *P. aphthosa*, namely amino groups, carboxylic groups of uronic acids, carboxylic groups of phenolic acids, and phenolic OH groups. They may participate in the ion-exchange reactions with the ions of the environment. The amount of ionogenic groups in *P. aphthosa* cell walls was found to depend on the zone and age of the thallus.

Key words: lichen, cell wall, mycobiont, ion-exchange properties, ionogenic groups.

DOI: 10.1134/S0026261709050178

Lichens are symbiotic organisms consisting of two or more genetically differentiated and closely associated physiologically and biochemically, heterotrophic fungus and a phototrophic microalga and/or cyanobacterium. In three-component lichens, cyanobacteria act as a diazotrophic component and are localized in the thallus as specialized structures (cephalodia) [1, 2]. In lichens, the fungal component (mycobiont) is usually an ascomycete or, less often, a basidiomycete; it plays a morphology-forming role. It constitutes 90 to 98% of the lichen biomass [1, 3].

Since lichens have no developed root system, mineral compounds are delivered to the thallus with atmospheric precipitation [3–5] in a liquid (rain, snow, fog, or dew) or solid state (aerosol, dust) [6]; they are absorbed by the whole thallus surface [7]. The cell walls of the mycobiont, phototrophic microalgae (photobiont), and cyanobacteria (cyanobiont) conceivably play the main role in this process [4, 8]. For example, nickel absorption by *Umbilicaria muehlenbergii* is a physicochemical process involving the carboxylic and oxycarboxylic acidic groups of the cell walls of mycobiont hyphae [9]. Some authors believe that chitin and the side groups of the protein molecules in the mycobiont cell walls play the main role in cation binding [10].

In the cell walls of plant tissues, ion-exchange reactions are known to change the composition of the apoplastic solution; an extracellular compartment thus participates in ion delivery to the cell. The ions leaving the cell also may bind to the cell wall functional groups; this process plays an important role in resistance of plant organisms to stress conditions. In lichens, potassium ions, which are removed from the mycobiont cells during drying, are sorbed on the active sites of the hyphal cell wall and return to the cells after humidification [10]. These data indicate an important role of ion-exchange processes in the lichen's cell walls. However, since lichen is a multicomponent system, the ion-exchange characteristics of the lichen's "cell walls" have not been determined. The mycobiont is the major component of lichen; ion-exchange properties of the thallus' cell walls are probably mostly determined by the characteristics of the cell walls of the mycobiont hyphae.

The goal of the present work was qualitative assessment of ion-exchange properties of the cell walls isolated from different zones of the thallus of the foliose lichen *Peltigera aphthosa* (L.) Willd and the content of ionogenic groups of all types participating in ion-exchange reactions.

MATERIALS AND METHODS

A three-component lichen *Peltigera aphthosa* (L.) Willd was the subject of the study. The material was collected in June 2006 in the vicinity of the N.A. Pertsov White Sea Biological Station of the Moscow State University (Rugozerka guba of the Kandalaksha Bay, White Sea). The thallus was cleared of soil particles, and the cephalodia (cyanobiont) were removed from the thallus surface. The thallus was then separated into three zones, apical (1 cm from the lobe margin), medial (2–4 cm from the lope margin, exceeding the apical zone), and basal (main mass of the thallus) (Fig. 1).

Cell wall isolation was carried out according to the procedure described previously [11, 12]. The samples were placed in a glass ion-exchange funnel (V = 250 ml)

[1] Corresponding author; e-mail: v.denis83@mail.ru

and washed under dynamic conditions sequentially with 1% NaOH (~0.5 l), distilled water (~2 l), 1% HCl (~0.5 l), and distilled water till the disappearance of the chloride ion, which was determined by titration with mercury nitrate. The material was then dried to a constant weight at 55–60°C in a desiccator with $CaCl_2$. This standardization procedure (transformation of all the cation-exchange groups in the cell wall's structure to the H^+ form and of all the anion-exchange groups, to the free amine form) made it possible to carry out comparative investigation of the ion-exchange characteristics of the isolated cell walls differing in the structure of their functional groups [11, 12].

Complete removal of the protoplast components from the isolated cell walls was monitored as described earlier [13] by fluorescence microscopy (Axioplan 2 imaging MOT, Zeiss, Germany). The preparations of the thallus and of the isolated cell walls were stained with 1 μg/μl solution of DAPI (4',6-diamidino-2-phenylindole; Sigma) in 50% ethanol at room temperature. DAPI binding to DNA results in a 20-fold increase of the fluorescence intensity. DAPI exhibits blue fluorescence (λ_{max} of excitation is 358 nm; λ_{max} of fluorescence, 451 nm). Microscopical investigation demonstrated the absence of isolated cell nuclei in the samples.

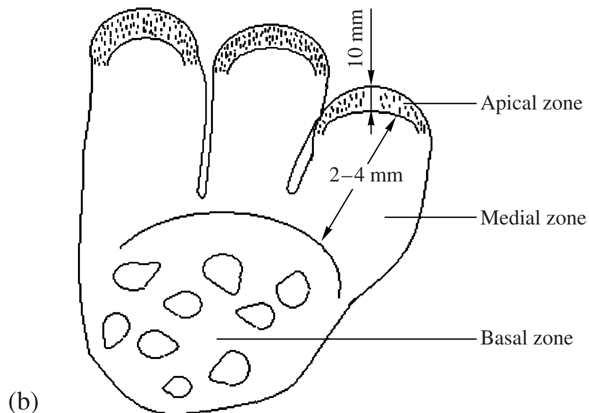
Determination of the qualitative and quantitative composition of ion-exchange groups was carried out by potentiometric titration of individual batches [11]. Dry weighted samples of 40 ± 0.1 mg were placed in plug-stoppered weighing bottles of ~ 50 ml and covered with 12.5 ml of a NaOH or HCl solution varying in concentration, but with the constant ionic strength of 10 mM created by addition of appropriate NaCl solutions. The range of acid or alkali concentrations in the original solutions varied from 0 to 10 mM. After 48 h, the samples were separated from the solution. Before and after contact with the samples, pH of the solutions was determined (Model 3320 pH Meter, Jenway, United Kingdom), as well as the concentration of acid or alkali (by titration with methyl red). The sorption capacity of the cell walls at a given pH was calculated from the changes in H^+ or OH^- concentrations in the solution using the equation:

$$S_i^{\mathrm{cat(an)}} = \frac{|(C_0 - C_i)| \times V}{g}, \quad (1)$$

where S_i is the ion-exchange capacity of preparations of the cell wall for cations (S_i^{cat}) or anions (S_i^{an}) at pH_i, μmol/g mass of the dry cell wall; C_0 and C_i are the relevant initial and equilibrium concentrations of NaOH or HCl in the solution, mM; V is the solution volume, ml; and g is the sample weight, g.

The titration curves were analyzed as described in [11, 12]. The amount of each type of ion-exchange groups (ΔS^j, j is the group type) was determined by ana-

(a)



(b)

Fig. 1. Morphological differentiation of the *P. aphthosa* thallus: aspect of the three-component foliate lichen (a) and the scheme demonstrating its apical, medial, and basal zones.

lyzing the experimental curves of dependence between the cell wall sorption capacity (S) and pH.

To calculate the ionization constants for the ionogenic groups, the Henderson–Hasselbach equation modified by Gregor [14] was used:

$$\mathrm{pH} = \mathrm{pK_a} + n\log_{10}\left(\frac{\alpha}{1-\alpha}\right), \quad (2)$$

where pK_a is the apparent ionization constant of the polymer's ionogenic group, α is the degree of dissociation, n is the constant depending on the composition of the polymer matrix and the nature of the counter-ion. By calculating the $\log_{10}\left(\frac{\alpha_i}{1-\alpha_i}\right)$ value for each pH

value and applying regression analysis, the pK_a^j and n^j values were determined for each step of ionization.

Using the values of the parameters (ΔS^j, pK_a^j, n^j), the rated relationship $S_i = f(pH_i)$ was determined for all experimentally obtained pH_i values according to the summary equation [14]:

$$S_i^{pac} = S_t^{cat} - \sum_{j,\, i=1}^{k,\, m} \Delta S^j \left[1 + 10^{\frac{(pK_a^j - pH_i)}{n^j}}\right]^{-1}, \quad (3)$$

where S_t^{cat} is the maximum ion-exchange capacity of the samples, ΔS^j is the number of ionogenic groups of type j, S_i^{calc} is the calculated ion-exchange capacity of the cell walls at a given pH_i value. S_t^{cat}, ΔS^j, and S_i^{calc} are expressed in μmol per gram of dry mass of the cell wall; pK_a^j is an apparent ionization constant for the j type ionogenic group, n^j is the constant of equation (2) for the j type ionogenic group, k is the number of points on the potentiometric curve, and m is the number of types of ionogenic groups.

The applicability of this approach to description of the acid–base balance was assayed by regression analysis; the parameters of the equation:

$$S_i^{calc} = BS_i^{ex} + A, \quad (4)$$

were determined, where S_i^{calc} and S_i^{ex} (μmol per gram of dry cell walls) are the experimental and calculated (from equation 3) ion-exchange capacity at a given pH_i value; A and B are the regression parameters. The calculations demonstrated that the model fits the experimental data precisely, as was evident from the correlation coefficients (r^{corr}) of the function $S_i^{calc} = f(S_i^{ex})$ and the A and B coefficients of equation (4). In all the variants, $r^{corr} \longrightarrow 1$, A does not exceed the experimental error, and $B \longrightarrow 1$.

Determination of water content in the thallus and of the weighting factor of cell wall swelling in water and solutions. The fragments of *P. aphthosa* thallus moistened in water for 30 min or the standardized cell walls swollen in water or solutions were dried with filter paper, and the dry mass was determined (G_F and G_F^{cw}, respectively). The thallus and cell wall samples were then dried to a constant weight at 60°C, and the dry mass was determined (G_D and G_D^{cw}, respectively). The weighting factor of swelling for the standardized cell walls (K^{cw}) and the water content in the thallus (Q) were determined from the equations [15]:

$$K^{cw} = \frac{G_F^{cw} - G_D^{cw}}{G_D^{cw}},$$

$$Q = \frac{G_F - G_D}{G_D},$$

where G_F and G_D are the mass of wet and dry samples, g; the *cw* index stands for the cell wall.

RESULTS AND DISCUSSION

The foliose lichen *P. aphthosa*, unlike other members of this genus, exhibits morphologically well-differentiated apical, medial, and basal zones of the thallus (Fig. 1). The apical zone is an actively growing edge of the thallus, with the hyphal tips on the upper surface protruding above the cortex layer. In the medial zone, small (1–2 mm in diameter) cephalodia are present, which are not submerged into the thallus and easily separated from the surface. Isolation of the cephalodia from the surface of the medial zone does not result in disruption of the mechanical structure of the thallus. In the basal zone, the cephalodia are big (up to 4 mm in diameter) and submerged into the cortex layer of the thallus. When the cephalodia are removed from the thallus surface, the cortex layer and the photobiont layer are not revealed.

The experimental dependences of $pH_i = f(S_i)$ for the cell walls of the foliose lichen *P. aphthosa* isolated from different parts of the thallus are presented on Fig. 2; S_i is the ion-exchange capacity of the cell walls at a given pH_i. The negative S values correspond to proton absorption by the ionogenic groups according to the equation $R–NH_2 + H^+ \longrightarrow R–\overset{+}{N}H_3$ and the positive S values, to proton release ($R - OH + Na^+ \longrightarrow R - ONa + H^+$). At pH > 10 and pH < 2, the sorption capacity for NaOH and HCl were the highest (S_t^{cat} and S_t^{an}, respectively). These values characterize the content of acidic (S_t^{cat}) and alkaline groups (S_t^{an}) in the polymer structure of the cell wall, which may potentially participate in exchange reactions at appropriate ambient pH values.

Our data suggest the presence of four types of ionogenic groups which are able to participate in ion-exchange reactions under appropriate conditions; three of them are cation-exchange groups, the fourth exchanges anions (Table 1). In the cell walls of the lichen the amino group content is 80–130 μmol per 1 g of dry mass of the cell wall, while the total content of the cation-exchange groups is from 500 to 565 μmol/g. In all the variants, the content of the cation-exchange groups significantly (four times or more) exceeds the content of the anion-exchange ones. These data indi-

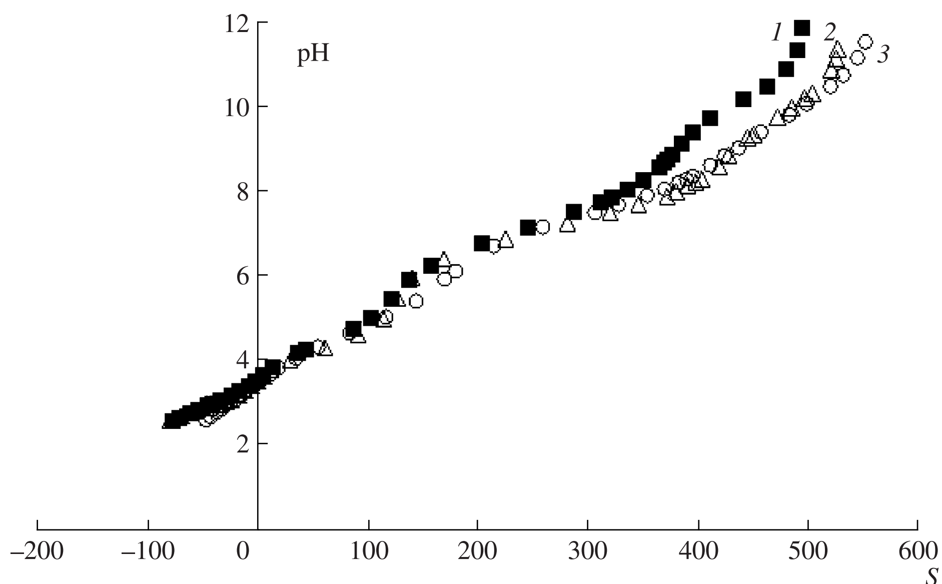


Fig. 2. Curves of potentiometric titration for the cell walls isolated from different zones of *O. aphthosa* thallus: apical (*1*), medial (*2*), and basal (*3*). *S* is the ion-exchange capacity of the lichen cell walls, μmol per 1 g dry cell wall mass.

cate that, similar to higher plants, cell walls of the lichen *P. aphthosa* have mainly cation-exchange properties and are therefore a natural cation exchanger [16].

According to the pK_a values (Table 2), data on the chemical composition of lichens [17], and pK_a values for various groups in low-molecular weight compounds [18], the region of the titrimetric curve at pH 9–10 may be assigned to titration of phenolic groups, since for phenol $pK_a = 9.98$ [18].

The results demonstrate (Fig. 3) that at pH 2.5–3.5, absorption of a proton, rather than its release occurs, i.e., ionization of the basic group occurs within this pH range. It can be therefore suggested that the groups with pK_a^1 ~2.7–2.9revealed in the cell walls of the lichen thallus are amino groups of glucosamine of chitin, which is among the components of the mycobiont's cell wall [19]. This conclusion agrees with the literature data on the structural heterogeneity of chitin and the presence of the links with free deacetylated amino groups [20–24]. The region of the potentiometric curve at pH 3.8–5.5 may be related to titration of the carboxylic groups of uronic acids of the photobiont.

Cation-exchange groups with pK_a ~ 7 pH units are most probably residues of phenolic acids (lichen compounds). This conclusion is supported by the following reasoning. Lichens produce complex specific organic compounds, especially polyphenolic acids (usnic acid, lecanoric acid, etc.) [25]. The ionization constant for the carboxylic group of cinnamic acid, which may be with certain assumptions considered a low-molecular analogue of polyphenolic acids, is 4.44 [18]. However, pK_a values of low-molecular and polymeric acids differ significantly. This may be illustrated by the following row of pK_a values changing with transition from low-molecular to three-dimensional polymeric structures: acrylic acid, 4.26 [18], polyacrylic acid, 4.8 [26], and its three-dimensional analogue, 5–7.5 [26] depending on the type and amount of the cross-linking agent. It may therefore be suggested with high probability that

Table 1. Quantitative composition of ionogenic groups in the cell walls isolated from different parts of the thallus of the lichen *P. aphthosa*

Thallus zone	$S_t^{an} = \Delta S^1$	ΔS^2	ΔS^3	ΔS^4	$S_t^{cat} = \Delta S^2 + \Delta S^3 + \Delta S^4$
Basal	80 ± 10	180 ± 16	220 ± 19	165 ± 13	565 ± 45
Medial	130 ± 12	120 ± 13	310 ± 25	100 ± 9	530 ± 40
Apical	100 ± 9	115 ± 10	270 ± 22	110 ± 9	495 ± 33

Note: S_t^{cat} and S_t^{an} are the cation- and anion-exchange capacity, respectively; *j* is the type of a group: amino groups (*1*), carboxylic groups of uronic acids (*2*), carboxylic groups of phenolic acids (*3*), and phenolic OH groups (*4*). ΔS^j, S_t^{cat}, and S_t^{an} are expressed in μmol per 1 g dry cell wall mass. The values are averages of three repeats and their standard deviations.

Table 2. Parameters of the acid–base balance in the cell walls isolated from different parts of the thallus of the lichen *P. aphthosa*

Thallus zone	*j*	pK_a^j	n^j	$r_{corr.}^j$	*k*	ΔS^j
Basal	*1*	2.78	–0.57	0.943	10	80
	2	4.69	1.07	0.951	7	180
	3	7.38	1.00	0.940	9	220
	4	9.73	1.60	0.876	12	165
Medial	*1*	2.68	–0.63	0.938	11	130
	2	4.28	0.60	0.966	6	120
	3	7.19	1.13	0.948	12	310
	4	9.84	1.04	0.798	9	100
Apical	*1*	2.91	–0.54	0.978	11	100
	2	4.43	0.75	0.966	6	115
	3	7.17	1.30	0.950	11	270
	4	10.06	0.89	0.944	6	110

Note: *j* is the type of a group; pK_a^j is the ionization constant of the *j*-th type group; n^j is the constant from equation (9) for the *j*-th type group; r_{corr}^j is the correlation coefficient for the *j*-th type group; ΔS^j is the content of the *j*-th type groups; k is the number of points in a line. Amino groups (*1*), carboxylic groups of uronic acids (*2*), carboxylic groups of phenolic acids (*3*), and phenolic OH groups (*4*). ΔS^j is expressed in µmol per 1 g dry cell wall mass.

the functional groups with pK_a ~7 in the three-dimensional structure of the cell walls are carboxylic groups of aromatic acids.

The cell walls isolated from different zones of *P. aphthosa* thallus did not differ in the qualitative composition of ionogenic groups, as was demonstrated by the values of the relevant ionization constants (Table 2). All the samples contain four types of ion-exchange groups, three cation-exchanges and an anion-exchange one.

The content of cation- and anion-exchange groups in the cell walls to a great degree depends on the thallus zone, i.e., on the age of the lichen. In the basal part of the thallus, the content of cation-exchange groups with the ionization constant pK_a^2 (ΔS^2, Table 2) is almost two times higher than in the apical zone. The highest content of anion-exchange groups (ΔS^1, Table 2) was found in the medial zone of the thallus. Moreover, the walls from the apical and medial zones of the thallus contain 1.4 times the number of cation-exchange groups with pK_a^3 than the walls of the basal zone.

In old and young parts of the thallus, the ratio of carboxylic and phenolic groups is different; the latter groups probably belong to lichen compounds. These results demonstrate that the qualitative and quantitative composition of lichen compounds changes with age; they are in agreement with the known data on the content of secondary metabolites in lichens depending on various factors, including age [27].

According to our data, the content of cation-exchange groups in cell walls of the lichens is lower than in the cell walls of plants. Most probably, apart from ion exchange, complex formation involving the nitrogen-containing components of the mycobiont's cell wall also occurs in the cell walls of the lichen. As was stated earlier, atmospheric sources are the main source of mineral nutrients for lichens [3]. Since the content of mineral compounds in the atmosphere is lower than in soil, the content of ion-exchange groups in the cell walls of the lichen is lower than in the plant cell walls.

Swelling is among the major physicochemical criteria for quantitative characterization of the ion-exchange properties of cell wall polymers. The swelling coefficient (K^{cw}), equal to the amount of water in a polymer per 1 g dry mass, is a quantitative characteristic of this process. Swelling of ion-exchange materials in aquatic solutions is caused by the presence of hydrophilic groups, while insolubility results from the presence of cross-links between the polymer molecules within the cell wall's structure. The degree of swelling for an ion-exchange material depends on the characteristics of the ionite and the composition of the solution. The higher the ratio of cross-links in the structure and the denser and harder the polymeric grid, the lower the swelling capacity.

The results obtained demonstrate that the mycobiont cells in the lichen possess relatively well-developed cell walls. In the total dry mass of thallus (*G*), their ratio is 40–60%; the value of this parameter depends on the zone (and thus on the age) of the thallus and increases from the apical zone to the basal one.

Table 3. Characterization of swelling of the thallus and cell walls of the lichen *P. aphthosa*

Thallus zone	Q	K_w^{cw}	K_{NaCl}^{cw}	G
Basal	1.85 ± 0.4	3.3 ± 0.7	2.20 ± 0.01	56 ± 5
Medial	1.6 ± 0.2	3.5 ± 0.7	1.95 ± 0.01	49 ± 3
Apical	1.5 ± 0.1	3.8 ± 0.7	1.88 ± 0.01	45 ± 2

Note: Moisture content in the thallus (Q), swelling coefficient of the cell walls in water (K_w^{cw}) and in 10 mM NaCl solution (K_{NaCl}^{cw}) are expressed in g H_2O per 1 g dry mass of the thallus and cell walls, respectively. G, % is the relative dry mass of the cell walls. The values are averages of 5–10 biological repeats and their standard deviations.

The swelling coefficient for the cell walls of the thallus in water (K_w^{cw}) varies from 3.3 (the basal zone) to 3.8 (the apical zone) g H_2O per 1 g dry biomass. The highest swelling coefficient was revealed in the apical zone of the thallus (Table 3). The differences may be due to the fact that the main processes of growth and thallus formation occur in the apical zone.

Thus, four ionogenic groups are present in the cell walls of the lichen *P. aphthosa*, namely amino groups, carboxylic groups of uronic acids, carboxylic groups of phenolic acids, and phenolic groups. These groups may participate in the reactions of ion exchange with the ambient ions. The content of ionogenic groups in the cell walls of *P. aphthosa* thallus was shown to depend on the age of the lichen; it was also found to be lower than in plant cell walls.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, project nos. 08-04-01398 and 08-04-00746-a.

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