ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 8, pp. 933-937. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © N. R. Meychik, Yu. I. Nikolaeva, I. P. Ermakov, 2009, published in Biokhimiya, 2009, Vol. 74, No. 8, pp. 1145-1151.

Nonaqueous Titration of Amino Groups in Polymeric Matrix of Plant Cell Walls

N. R. Meychik*, Yu. I. Nikolaeva, and I. P. Ermakov

Biological Faculty, Lomonosov Moscow State University, 119899 Moscow, Russia; fax: (495) 939-4309; E-mail: meychik@mail.ru

Received June 2, 2008
Revision received October 17, 2008

Abstract—Nonaqueous titration was used for detection of free amino groups in the polymeric matrix of plant cell walls. The content of amino groups varied in the range 0.54-0.91 and total nitrogen in the range 1.0-4.2 mmol per gram dry mass of cell walls depending on the plant species. However, these data on the high content of free amino groups do not correlate with the present day concept that the nitrogen fraction in charged amino groups in plant cell wall proteins, which are assumed to be mainly amino groups of lysine and arginine residues, is about 10%. It is supposed that most detected free amino groups belong to the hydroxy-amino acids hydroxyproline and tyrosine that can be bound at the hydroxyl group with the carbohydrate part of glycoprotein or another structural cell wall polymer.

DOI: 10.1134/S0006297909080161

Key words: amino groups, cell wall, plant roots

Plant cell wall is a delicately organized intricate complex of various polysaccharides, proteins, and aromatic substances [1] that changes structurally and functionally during cell life [2]. The protein component of the cell wall matrix is the most difficult to study because all available data about its composition and structure are based on investigation of hydrolysates and extracts obtained by the treatment of isolated cell wall with enzymic and/or electrolytic reagents [3-8].

Cell walls contain structurally different proteins including many enzymes, especially oxidoreductases (peroxidase, ascorbate oxidase, polyphenol oxidase, polyamine oxidase, malate dehydrogenase, etc.) and hydrolases (exo- and endoglycanases, pectin methylesterase, protease, etc.) [5, 6]. However structural proteins devoid of enzymic activity but involved in formation of the cell wall matrix are prevalent. Four groups of structural proteins are distinguished in cell walls of higher plants: extensins, arabinogalactan, and proline-enriched and glycine-enriched proteins. Extensins and hydroxyproline-enriched proteins are predominant, their amount reaching 90% of total content of all cell wall proteins [9]. These proteins are glycoproteins, 50-65% of which are carbohydrate, and 97% of the latter is arabinose and 3% is galactose. They are covalently bound with non-protein cell wall components, and it is impossible to recover them from matrix without destruction of other polymeric components. The amount of structural protein in cell walls can be judged from the content of total nitrogen and free amino groups, which are thought to be mainly side radicals of lysine and arginine residues. It has been shown that the latter make up about 10% of total nitrogen [5, 6]. There is practically no information about content of amino groups in the cell wall structural polymers, and methods and approaches to solution of this analytical problem are not yet developed. At the same time, data on qualitative estimation of nitrogen-containing polymers, obtained without using destructive methods, should help in understanding important aspects of the cell wall structural and functional peculiarities.

This work deals with detection of amino groups in the cell wall matrix of different plant species and estimation of the role of these groups in maintenance of matrix structure.

MATERIALS AND METHODS

The roots of following plants were used in this work: 38-day-old wheat plants (*Triticum aestivum* L., var. Inna), 60-day-old plants *Suaeda altissima* (L.) Pall., and spinach *Spinacia oleracea* L., var. Matador, 20-day-old plants of

* To whom correspondence should be addressed.

Oregon pea (*Vigna radiata* L.), chick pea (*Cicer arietinum* L., var. Bivanij), and vetch (*Vicia narbonesis* L., line Sel2384). Wheat, Oregon pea, chick pea, and vetch plants were grown on Pryanishnikov medium [10], while suaeda and spinach plants were grown on Robinson medium [11].

The root cell walls were isolated according to a previously described technique [12, 13]. The excised roots were placed in a glass ion-exchange column (V = 250 ml), sequentially washed under dynamic conditions with 1% alkali, acid, and distilled water until chloride was no longer found in the wash waters, then dried to constant weight in the presence of adsorbent ($CaCl_2$) at 55-60°C. The specified method of standardization, i.e. conversion of all cation-exchange groups present in the cell wall structure into H^+ form, allows comparison of the sorption properties of samples with different structure of functional groups [12].

Completeness of isolated cell wall purification of protoplast components was monitored by a previously described method [14] using fluorescence microscopy.

Completeness of cell wall purification of cytoplasmic proteins and those from other compartments was checked by protein determination by Lowry in supernatant after incubation of standardized ground cell wall preparations in 1% SDS solution at 25°C for two days.

Amino groups in low molecular weight compounds and albumin were determined using the method described in [15]. A weighed portion of a nitrogen-containing sample (10 ± 0.1 mg) was placed in a 50 ml flask and dissolved in 10 ml glacial acetic acid. When necessary, the solution was kept in a thermostat at 40°C until full dissolution of the substance. After cooling at room temperature, an aliquot of the solution (V_a) was placed in a conical flask and titrated with ~10 mM perchloric acid solution in glacial acetic acid in the presence of indicator 0.1% crystal violet in glacial acetic acid until the violet color changed to blue. In the presence of halides in samples, 3 ml of 3% mercuric acetate was added to the solution before titration. The $HClO_4$ concentration in glacial acetic acid was detected using a known concentration potassium biphthalate solution in glacial acetic acid. The concentration of nitrogen-containing compound (C_N, mM) was calculated according to formula:

$$C_N = (V_{HCLO_4} \cdot N_{HCLO_4} \cdot V_0)/V_a, \quad (1)$$

where V_{HClO_4} and N_{HClO_4} are the perchloric acid volume (ml) spent for titration of V_a ml solution of nitrogen-containing substance and its concentration (mM), respectively; V_0 is the volume (ml) of glacial acetic acid used for dissolving the weighed portion of nitrogen-containing compound.

Detection of amino group content in chitin and chitosan (ICN, USA) and in the cell wall polymeric matrix. A weighed portion of ground and dry preparation (20 ± 0.1 mg) was suspended in 7 ml of 10 mM $HClO_4$ solution in glacial acetic acid. Two days later the sample specimens were separated from the solution. Before and after contact with preparations, the solution was titrated with potassium biphthalate solution in glacial acetic acid in the presence of crystal violet indicator. The content of amino groups in samples was calculated on the basis of changes in perchlorate ion concentrations in initial and final solutions (N_{NH_2}, μmol per gram of the preparation dry mass) using the formula:

$$N_{NH_2} = ((V_{init} - V_{fin}) \times N_{Kbiphth} \times V_{tot})/(V_a \times g), \quad (2)$$

where V_{init} and V_{fin} are the amount of potassium biphthalate used for titration of initial and final (after contact with preparations) solution (ml); $N_{Kbiphth}$ is normality of potassium biphthalate (mM); V_a, the amount of solution used for titration (ml); V_{tot}, total volume of solution used for suspension of the weighed portion (ml); g, the sample weighed portion (g).

Fraction of the cell wall dry mass (G) in the tissue dry mass was determined by gravimetry. Two identical root portions were weighed. The first was fixed for 5 min at 100°C, dried at 55-60°C to constant weight, and the dry mass of the sample was determined (G_{tis}). Cell wall was isolated from the other and dried to constant weight (G_{cw}). The fraction of the cell wall dry mass (G) in the tissue dry mass was determined by formula:

$$G = (G_{cw}/G_{tis}) \times 100. \quad (3)$$

Elemental content of samples preliminarily ground and dried to constant weight at 55-60°C, was determined in the Laboratory of Elemental Analysis of Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences. Plant material subjected to catalytic combustion at 1000°C with subsequent separation of reaction products on a gas chromatography column was analyzed using an automatic CNH analyzer (Carlo-Erba, Italy).

Statistical data processing was done using the Excel program. Mean values and their standard deviations are shown in tables.

RESULTS AND DISCUSSION

Control determination of content of amino groups in low molecular weight nitrogen-containing compounds including amino acids showed that the content of amino groups in 1 mol, determined by nonaqueous titration, is equal to that calculated according to the structural formula of each individual compound (Table 1). Similar measurements in chitin and chitosan samples with known nitrogen and free amino group content, as well as in albumin, showed that the chosen method of analysis can be used for cross-linked structures: chitosan data obtained

Table 1. Experimental (Q_{exp}) and calculated (Q_{calc}) values corresponding to amino group content in 1 mol of substance

Compound	Q_{calc}	Q_{exp}
Glycine	1	1.039 ± 0.012
Arginine-HCl	2	2.088 ± 0.096
Lysine-HCl	2	2.066 ± 0.117
Phenylalanine	1	1.018 ± 0.036
Proline	1	1.020 ± 0.033
Betaine, anhydr.	1	0.915 ± 0.13
Betaine·HCl	1	1.079 ± 0.080

Note: Mean values obtained in 7-10 independent experiments and their standard deviations are shown.

Table 2. Content of free amino groups (N_{NH_2}), total nitrogen (N), and fraction of free amino groups ((N_{NH_2}/N) × 100)) in chitin, chitosan, and albumin

Specimen	N_{NH_2}	N	$(N_{NH_2}/N) \times 100$
Chitin	1295 ± 84	5057 ± 50	27
Chitosan	680 ± 33	650 ± 25	104
Albumin	1330 ± 100	11 500	11

Note: N_{NH_2} and N are expressed in μmol nitrogen per gram of sample dry mass, ((N_{NH_2}/N) × 100) in %. N is calculated on the basis of elemental analysis according to the formula $N = N(\%)/1.4$. Mean values of three independent experiments and their standard deviations are shown.

by nonaqueous titration and results of elemental analysis are in complete agreement (Table 2). This conclusion is also confirmed by data of chitin and albumin analysis. The fraction of free amino groups in the first is 27% and in the second 11% (Table 2). Results of chitin analysis for content of non-acetylated amino groups completely correspond to data of other researchers [16], whereas results of albumin analysis correlate with data on the content in proteins of free amino groups (~10%) belonging to charged arginine and lysine residues [5, 6].

Results of nonaqueous titration of the cell wall standardized preparations show that they contain 0.54-0.91 mmol amino groups per gram of cell wall dry mass depending on the plant species; this is 21-53% of total nitrogen in the extracellular compartment ((N_{NH_2}/N_{cw}) × 100) (Table 3).

To compare N_{NH_2} values with total nitrogen content in isolated cell walls and whole roots, elemental analysis of these preparations was carried out (Table 4). Total nitrogen content in the root tissues (N_{tis}) and in cell walls isolated from roots (N_{cw}) varies in the range 3-9 and 1.5-5.8%, respectively (Table 4).

Correct comparison of N_{tis} and N_{cw} values requires determination of the cell wall dry mass fraction (G) in dry mass of the whole tissue: values of this parameter are largely dependent on the plant species. In spinach, the G value does not exceed 28%, whereas in wheat and suaeda it reaches 56-57% (Table 4).

Calculations show that the fraction of the cell wall nitrogen in total nitrogen content in root tissues (D_{cw}) is 17-37% depending on the plant species (Table 4).

Talmadge et al. [17] estimated protein content in the cell wall polymeric matrix (G_{prot}) on the basis of total nitrogen (N_{cw}) analysis using the following formula for calculation: $G_{prot} = N_{cw} \times 6.25$.

Similar calculations based on results of this work show that protein content (G_{prot}) in cell walls varies in different plants in the range of 9-37% of mass of the cell wall polymers (Table 4). However, absolute G_{prot} values, based on data of elemental analysis, are higher than those obtained by different authors using different methods for protein content determination in cell walls. Thus, proteins of insoluble cell wall fraction isolated from the bean

Table 3. Content of free amino groups (N_{NH_2}) and total nitrogen (N_{cw}) in isolated cell walls

Plant	N_{NH_2}	N_{cw}	N^*_{prot}	$(N_{NH_2}/N_{cw}) \times 100$	N^*_{prot} (%)	G^*_{prot}
Chick pea	0.71 ± 0.01	3.33	2.91	21	4.08	25
Vetch	0.913 ± 0.08	4.18	3.63	22	5.08	32
Oregon pea	0.658 ± 0.05	2.31	1.84	29	2.57	16
Wheat	0.54 ± 0.04	1.04	0.56	53	0.78	4.9
Spinach	0.832 ± 0.06	3.05	2.46	27	3.45	22
Seablite (suaeda)	0.652 ± 0.05	1.77	1.24	37	1.74	11

Note: N^*_{prot}, ((N_{NH_2}/N_{cw}) × 100), N^*_{prot} (%), and G^*_{prot} are protein nitrogen content, fraction of amino group nitrogen in total nitrogen content, protein nitrogen, and protein percentage in isolated cell walls. N_{NH_2}, N^*_{prot}, and N_{cw} are expressed in mmol per gram cell wall dry mass. N_{NH_2} determined by nonaqueous titration of isolated cell walls. N_{cw} is calculated on the basis of elemental analysis using formula: $N_{cw} = N_{cw}(\%)/1.4$, where $N_{cw}(\%)$ is percentage of total nitrogen in cell walls. Values N^*_{prot}, N^*_{prot} (%), and G^*_{prot} are calculated in accordance with the formulas: $N^*_{prot} = (N_{cw} - N_{NH_2})/0.9$; N^*_{prot} (%) $= N^*_{prot} \times 1.4$; $G^*_{prot} = 6.25 \times N^*_{prot}$ (%).

Table 4. Total nitrogen content in tissues (N_{tis}, %) and isolated cell walls (N_{cw}, %). G, D_{cw}, and G_{prot} are fraction of cell wall dry mass in dry mass of root tissues, fraction of the cell wall nitrogen in total nitrogen of root tissues, and protein content in cell wall (%), respectively

Plant	N_{tis}, %	N_{cw}, %	G	D_{cw}	G_{prot}
Chick pea	9.09 ± 0.15	4.66 ± 0.10	39 ± 4	20	29
Vetch	8.34 ± 0.11	5.85 ± 0.05	43 ± 4	30	37
Oregon pea	7.74 ± 0.09	3.23 ± 0.04	40 ± 3	17	20
Wheat	4.51 ± 0.010	1.45 ± 0.03	57 ± 3	18	9
Spinach	3.25 ± 0.05	4.27 ± 0.08	28 ± 2	37	27
Seablite (suaeda)	3.71 ± 0.08	2.48 ± 0.05	56 ± 3	37	16

Note: N_{tis} and N_{cw}, data of elemental analysis, %; G, values determined experimentally; D_{cw} and G_{prot}, values calculated according to formulas: $D_{cw} = (N_{cw} \times G)/N_{tis}$ and $G_{prot} = 6.25 \times N_{cw}$.

seed lobes made up 26% of total content of polymers in this compartment [4]. The high protein content in cell walls can be due to the presence in preparations of contaminating cytoplasmic proteins [7, 8]. However, analysis in our laboratory has shown that in the cell wall preparation contaminations of cytoplasmic proteins and proteins from other compartments do not exceed 1-2.5% of total amount of tightly bound cell wall proteins. Thus, it can be concluded that relatively high protein content in cell wall of the studied plants is not due to the presence in the preparations of contaminations of cytoplasmic proteins and proteins from other cell compartments.

Our data on the high content of free amino groups (Table 3) do not correlate with the present day concepts that charged amino groups of the plant cell wall proteins, represented mainly by side radicals of lysine and arginine residues, make up about 10% of protein mass.

There is practically no information in the literature concerning direct quantitative determination of total nitrogen and nitrogen-containing compounds of protein and non-protein types in isolated cell wall. The available information on the composition and quantity of its structural proteins is based on results of chemical and physicochemical analysis of extracts or hydrolysates obtained by treatments of cell walls by salt, acid, and alkali solutions or by enzymes at various temperatures [5-8, 17]. We have investigated the cell wall polymeric matrix as an entire apoplast component, and in this case each sample was studied by cytological methods to confirm the purity of isolated preparations from cytoplasmic contamination. It should be pointed out that in addition to the amino acid group, nitrogen of heterocycles, amine salts, and quaternary ammonium bases, nonaqueous titration in acetic acid can also determine alkaline salts of carbonic acids. However, standardization technique was used during cell wall isolation, i.e. all carboxylic groups of carbonic acids, present in the cell wall structure, were converted to H^+ form, which allows one to exclude the supposition that salts of carbonic acids are present in functional groups determined by nonaqueous titration. Thus, results of this investigation suggest that functional groups in the cell wall polymeric matrix, revealed by non-aqueous titration, are nitrogen-containing groups.

Since amino groups are highly basic (pK_a > 10-11), we suppose that the amino groups determined by us are mainly hydroxyamino acids (tyrosine, hydroxyproline) joined to the cell wall polymers via the hydroxyl group. This supposition is based on the following arguments and experimental data. We showed previously that in plants of different species upon titration of the cell wall polymeric matrix in aqueous medium there is no proton release in the pH range <3.5, but, on the contrary, their absorption, i.e. in this pH region ionization of basic groups takes place in accordance with reaction $\sim RNH_2 + HCl \rightarrow [RNH_3]^+Cl^-$ [12, 13], and their amount does not exceed 80-140 μmol per gram cell wall dry mass. It can be assumed that groups detected in this pH region are amino groups of the cell wall polymeric structure, because cell wall does not contain different basic groups. It should be especially emphasized that the content of ionized basic groups increases as acidity of the medium increases. These results agree with data of other researchers. Thus, it was shown that the anion exchange ability of the cell wall isolated from *Sphagnum russowii* is exhibited in acidic region and in aqueous medium it makes up 60-66 μmol per gram cell wall dry mass [18]. The authors showed that these groups are amino groups but they did not put forward any ideas concerning the nature of the group.

In the pH range 3-11 amino acid residues of hydroxyamino acids are in intra-salt form and have no charge, because pK_a of the carboxylic group and amino group of amino acid residues are beyond the limits of this pH region. According to pK_a of hydroxyamino acids, carboxyl protonation begins at pH < 3.0-3.5, intra-salt form is destroyed, and amino groups gain positive charge. Just owing to this, cell wall anion exchange ability is revealed at HCl concentration in the medium >0.5 mM [12, 13]. These suppositions are supported by functional analysis

of the cell wall polymeric matrix, results of which are given in Table 3. We have shown that the content of amino groups in polymeric matrix significantly exceeds that based on titration in aqueous medium. This is possible only if the amino groups revealed by us in neutral, weakly alkaline, or weakly acid medium are in real intra-salt form.

Presently it is proved that the tyrosine di-, tri, and tetramers (isodityrosine, pulkerosine, and diisodityrosine, respectively) are involved in formation of rigid intermolecular bonds between cell wall proteins, mainly between extensins, or between proteins and other structural components of the matrix [19]. Just formation of such bonds is considered by researchers as factors responsible for high resistance of the cell wall structural proteins to enzymic and electrolytic reagents [5, 6, 19]. It was also shown by NMR and mass spectrometry that in diisodityrosine two isodityrosine molecules are joined via diphenyl bonds [19, 20]. These data and results of this work suggest the presence in the cell wall matrix of free amino acid fragments belonging to tyrosine involved in formation of intermolecular bonds but not included in protein composition, i.e. not involved in peptide bond formation.

It is known that the hydroxyproline fraction in cell wall hydrolysates can reach 45% of total content of amino acids [5, 6, 17]. Besides hydroxyproline-enriched proteins, 2% of hydroxyproline, bound to tetraarabinosides via the hydroxyl group, was found in isolated cell wall of *Acer pseudoplatanus*. These data and results of our work indicate the presence in the cell wall matrix of amino acid fragments belonging to hydroxyproline and bound at the hydroxyl group with the cell wall carbohydrate components.

Thus, comparison of our results on the total nitrogen and amino group content with data from the literature indicate that most free amino groups determined by nonaqueous titration are hydroxyamino acids (hydroxyproline and tyrosine) that may be bound at the hydroxyl group with the carbohydrate part of the glycoprotein or a different structural polymer of the cell wall.

We have used data of elemental and functional analysis to recalculate protein content in the cell wall (G_{prot}, Table 3) with regard that only 10% of amino groups may belong to these proteins. Data obtained in such a way well agree with results of other authors on total protein content in cell walls (0.1-30% dry mass) [9]. It is also known that it is significantly higher in cell walls of dicotyledons compared to monocotyledons [5, 6], and this correlates with our data (Tables 3 and 4).

Thus, nonaqueous titration of free amino groups within cell walls of plant roots revealed some previously unknown structural peculiarities of their nitrogen-containing polymers: cell walls are characterized by high content of free amino groups (20-50%), which significantly exceeds that in proteins (~10%). This can be explained by the fact that plant cell walls contain amino acid residues not involved in peptide bond formation and belonging to hydroxy-amino acids that are bound at the hydroxyl group with the carbohydrate part of glycoprotein or another structural polymer of cell walls.

This work was supported by the Russian Foundation for Basic Research (grants 04-04-49379-a and 08-04-01398-a).

REFERENCES

1. Fry, S. C. (2004) *New Phytologist*, **161**, 641-675.
2. Gorshkova, T. A. (2007) *Plant Cell Wall as Dynamic System* [in Russian], Nauka, Moscow.
3. Zeier, J., Goll, A., Yokoyama, M., Karahara, I., and Schreiber, L. (1999) *Plant, Cell Environ.*, **22**, 271-279.
4. Shiga, T. M., Lajolo, F. V., and Filisetti, T. M. C. C. (2003) *Cienc. Technol. Aliment.*, **23**, 141-148.
5. Gassab, G. I. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 281-309.
6. Gassab, G. I., and Varner, J. E. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **39**, 321-353.
7. Feiz, L., Irshad, M., Pont-Lezica, R. F., Canut, H., and Jamet, E. (2006) *Plant Method*, **2**, 10-22.
8. Jamet, E., Canut, H., Boudart, G., and Pont-Lezica, R. F. (2006) *Trends Plant Sci.*, **11**, 33-39.
9. Sharova, E. I. (2004) in *Plant Cell Wall*, St. Petersburg State University Publishing, St. Petersburg.
10. Baslavskaya, S. S., and Trubetskova (1964) in *Practical Course on Plant Physiology* [in Russian], Moscow State University Publishing, Moscow.
11. Robinson, S. P., and Dountov, S. D. (1985) *Austr. J. Plant Physiol.*, **12**, 471-479.
12. Meychik, N. R., Ermakov, I. P., and Savvateeva, M. V. (1999) *Plant Physiol. (Moscow)*, **46**, 742-747.
13. Meychik, N. R., and Yermakov, I. P. (1999) *Plant & Soil*, **217**, 257-264.
14. Meychik, N. R., Matveeva, N. P., Nikolaeva, Yu. I., Chaikova, A. V., and Yermakov, I. P. (2006) *Biochemistry (Moscow)*, **71**, 893-899.
15. Cheronis, N. D., and Ma, T. S. (1973) in *Micro- and Semimicromethods of Organic Functional Analysis* [Russian translation], Khimiya, Moscow.
16. Feofilova, E. P. (1983) in *Cell Wall of Fungi* [in Russian], Nauka, Moscow.
17. Talmadge, K. W., Keegstra, K., Bauer, W. D., and Albersheim, P. (1973) *Plant Physiol.*, **51**, 158-173.
18. Richter, C., and Dainty, J. (1989) *Can. J. Bot.*, **67**, 460-465.
19. Brady, J. D., Sadler, I. H., and Fry, S. C. (1996) *Biochem. J.*, **315**, 323-327.
20. Brady, J. D., Sadler, I. H., and Fry, S. C. (1998) *Phytochemistry*, **47**, 349-353.