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Investigations on the Protein Composition of the Lichen *Pseudevernia furfuracea* (L.) Zopf var. *ceratea* (ACH.) HAWKSW. from Different Altitudes

By

Anna STROBL*), Roman TÜRK*) & Josef THALHAMER**)

With 6 Figures

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Summary

STROBL A., TÜRK R. & THALHAMER J. 1994. Investigations on the protein composition of the lichen *Pseudevernia furfuracea* (L.) ZOPF var. *ceratea* (ACH.) HAWKSW. from different altitudes. – Phyton (Horn, Austria) 34 (1): 67–84, 6 figures. – English with German Summary.

The protein composition of the fruticose lichen *Pseudevernia furfuracea* var. *ceratea* was investigated by SDS-PAGE, immunoblotting, and two-dimensional gel electrophoresis. Minor differences of protein compositon were observed between the individuals of a habitat, but they were negligible in comparison to the variations between different habitats. The altitude of the habitat exerted an influence upon total protein content, in a study comparing heights above sea level between 50 m and 1800 m. Using SDS-PAGE it could be shown that increasing altitude resulted in a decreased amount of the 17 kD, 29 kD, 48 kD, 64 kD, and 73 kD proteins. Only the band at 14 kD was stronger at 1800 m than at lower sites.

Immunoblotting likewise revealed a dependence of protein composition on altitude, although the rabbit antisera showed only poor immunological activity against the lichen proteins. A band at 66 kD was identified by antibodies in lichens found at 1000 m, 750 m, 300 m, and 50 m but not at 1100 m, 1400 m, or 1800 m.

A classification of habitats into one group situated above 1000 m and another situated below 1000 m could be concluded from the results of two-dimensional gel

*) Mag. Dr. A. STROBL, Dr. R. TÜRK, Institut für Pflanzenphysiologie, Universität Salzburg, Hellbrunnerstraße 34, A-5020 Salzburg (Austria).

**) Dr. J. THALHAMER, Institut für Biochemie, Universität Salzburg, Hellbrunnerstraße 34, A-5020 Salzburg (Austria).

electrophoresis. Although there was qualitative and quantitative variation between individual proteins, the statistical presence of the protein spots permitted the division into one class with the habitats at 1800 m, 1400 m, 1100 m, and 1000 m and a second class consisting of those at 750 m, 300 m, and 50 m.

Zusammenfassung

STROBL A., TÜRK R. & THALHAMER J. 1994. Untersuchungen des Proteinmusters der Flechte *Pseudevernia furfuracea* (L.) ZOPF var. *ceratea* (ACH.) HAWKSW. in Abhängigkeit von der Höhenstufe. – Phyton (Horn, Austria) 34 (1): 67–84, 6 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Das Proteinmuster der Strauchflechte *Pseudevernia furfuracea* var. *ceratea* wurde mit SDS-PAGE, Immuno-Blotting und Zweidimensionaler Gelelektrophorese untersucht. Innerhalb der Individuen eines Standortes waren geringfügige Proteindifferenzen zu beobachten, die aber, verglichen mit den Abweichungen bei verschiedenen Standorten, vernachlässigbar waren. Die Seehöhe des Standortes – Höhenlagen von 50 m bis 1800 m wurden verglichen – beeinflußte das Gesamtprotein von *Pseudevernia furfuracea* var. *ceratea*. Anhand von SDS-PAGE konnte belegt werden, daß mit steigender Seehöhe ein Rückgang der Proteine bei 17 kD, 29 kD, 48 kD, 64 kD und 73 kD eintritt. Lediglich die 14 kD-Bande war auf 1800 m proteinreicher als auf den tiefer gelegenen Probenahmeorten.

Mit Immuno-Blotting wurde ebenfalls eine Höhenabhängigkeit des Proteinmusters nachgewiesen. Eine Bande bei 66 kD wurde in den Proben der Standorte auf 1000 m, 750 m, 300 m und 50 m von Antikörpern erkannt, nicht aber auf 1100 m, 1400 m und 1800 m.

Eine Unterteilung der Standorte in eine über 1000 m und eine unter 1000 m gelegene Gruppe konnte aus den Ergebnissen der Zweidimensionalen Gelelektrophorese abgeleitet werden. Trotz der Variationen einzelner Protein-Spots in Qualität und Quantität wies die Abundanz der Gesamtheit der Proteine eine Klasse mit den Flechten der Standorte auf 1800 m, 1400 m, 1100 m und 1000 m sowie eine zweite Klasse mit jenen aus den Höhen 750 m, 300 m und 50 m aus.

1. Introduction

For investigations on the protein composition the lichen *Pseudevernia furfuracea* var. *ceratea* was chosen because of its widespread distribution (from Scandinavia to southern Italy) and because of its wide ecological range. On suitable substrate it is able to adapt on numerous environmental influences and to compete successfully for the respective habitat. The environmental factor that we chose for closer investigations was the height above sea level of the habitat. Various climatic and other factors are connected to its variation: the intensity of radiation increases with higher altitudes as well as the precipitation, the influence of UV light, and the stress caused by ozone. Additionally the growing season is shortened and the average temperatures are lower. These and other stressors cause a complex network of interfering actions and reactions, that we wanted to analyze on

the basis of the protein composition of the lichen. The methods we used were SDS-PAGE, immunoblotting, and two-dimensional gel electrophoresis.

Electrophoretic techniques for protein and enzyme analysis have already been proved successfully in discovering developmental differences (VERKLEY & ZUETENHORST 1980) as well as changes caused in plants by environmental stresses (SKULT & al. 1990, KRASNUK & al. 1978 a, b, MCCOWN & al. 1969, ORUS & ESTEVEZ 1984, FAHSELT & KROL 1989, DE JONG 1973, SIEFFERT & QUEIROZ 1989, PERRAS & SARHAN 1989, GUY & HASKELL 1989, MARMIROLI & al. 1986, 1989). The present studies should clarify the question if the adaption of an organism to changing environmental conditions is reflected in its protein composition or if the physiological regulation occurs on another level, for example different enzyme kinetics.

2. Material and Methods

2.1. Material and Habitat

The lichen *Pseudevernia furfuracea* (L.) ZOPF var. *ceratea* (ACH.) HAWKSW. was collected from several habitats at different altitudes. The collecting sites were

- at about 1800 m (Austria, Salzburg, Mühlbach/Dienten am Hochkönig, 500 m South of the Kollmannsegg, on *Picea abies*),
- at about 1500 m (Austria, Salzburg, Werfenweng, some meters below the Strussing-Hütte, on *Picea abies*),
- at about 1400 m (Austria, Salzburg, Dientener Sattel, on *Picea abies*),
- at about 1100 m (Italy, Calabria, Cannigliatello, Silano, on *Pinus laricio,* leg. D. PUNTILLO),
- at about 1000 m (Austria, Salzburg, Bischofshofen, some meters above the Ronachgut, on *Picea abies*),
- at about 750 m (Austria, Salzburg, Bischofshofen, some meters above the Triglgut, on *Picea abies*),
- at about 300 m (Great Britain, Scotland, Wigtonshire, Black Craig of Dee, on granite rocks), and
- at about 50 m (Sweden, 15 km East of Lund, Sandby par. Eliselund, on *Fraxinus excelsior,* leg. I. KÄRNEFELT).

To keep the seasonal variations of the protein composition as small as possible all collections were done during spring and early summer.

2.2. Methods

2.2.1. Collection of the Samples

The lichens were carefully cleaned from debris and their lobe tips were cut at a length of 1.0–1.5 cm. Only these apical thallus parts were used for further preparation to avoid developmental differences in the lichen. The material was air-dried and then stored at –21°C or used immediately. As many separate thalli were

used for one protein extraction course an intense mixture of different individuals of the population was guaranteed; thus intraspecific differences were no disturbing factors.

2.2.2. Preparation of the Protein Extracts

2.2.2.1. Protein extraction for SDS-PAGE and immunoblotting

The protein extraction was carried out according to FAHSELT 1980, 1986, FAHSELT & JANCEY 1977, FAHSELT & HAGEMAN 1983, and MARTIN 1977. The thalli were washed five times in acetone (each washing 150 ml/g air-dried lichens) to remove the extracellular phenolic substances. Then they were air-dried and ground to a fine homogeneous powder with liquid nitrogen using mortar and pestle. The cells were cracked in a glass bead homogenizer where the total protein from 0.5 g of the lichen powder was extracted with 10 ml of phosphate buffer (0.06 M NaH_2PO_4, 1 mM 2-mercaptoethanol, pH 6.6). The disintegrated material was sucked through a glass sinter and additionally washed with 5 ml of phosphate buffer. Sodium azide to a final concentration of 0.1% was added to protect the protein extracts from bacterial decay. For an increase in protein concentration the extracts were dried over silicic acid at 4° C for several hours. Afterwards the protein assay from BIORAD showed a protein concentration between 1.5 mg/ml and 3.0 mg/ml with BSA used as a standard. These raw protein extracts were stored frozen.

2.2.2.2. Protein extraction for the Two-Dimensional Gel Electrophoresis

0.5 g of lichen powder were incubated in 5 ml of lysis buffer (9.8 M urea, 20% triton X-100, 20% ampholyte 7–9 (BIORAD), 100 mM 2-mercaptoethanol) at 4° C for several hours. Then the suspension was warmed to 37° C and sonicated. Ensuing a triple change of freezing and thawing was done to accomplish a proper cell disruption. The preparations were centrifuged at 370 g for 10 minutes and the proteins were precipitated from the very viscous supernatant by adding ammonium sulphate to 80% saturation. The obtained raw protein was resuspended in 0.06 M phosphate buffer, pH 6.6 and dialyzed against bidistilled water. For an increase in protein concentration the extracts were dried for several hours over silicic acid. Then they were stored frozen in small portions at –21° C.

2.2.3. SDS-PAGE

The separation of the total lichen protein was carried out in a discontinuous glycin-tris buffer system according to LAEMMLI 1970 and DAVIES 1964. The gels used were gradient gels with 7.5–20% acrylamide-bisacrylamide (29 : 1), 0.2% SDS, 0.375 M tris, ph 8.8. For polymerization the gels contained 0.05% TEMED (N,N,N',N'-tetramethylethylenediamin) and 0.033% ammonium persulphate as catalysts. The gel size was 5.5 cm × 8.0 cm × 0.1 cm. A stacking gel with 4% acrylamide-bisacrylamide (29 : 1), 0.2% SDS, 0.125 M tris, pH 6.8, 0.1% TEMED, and 0.08% ammonium persulphate was layered over the separation gel. By inserting a teflon comb into the stacking gel during polymerization wells were provided for the application of the protein samples.

The lichen protein extracts were mixed 1 : 1 with sample buffer (100 mM 2-mercaptoethanol, 5% SDS, 10% glycerol, 0.06 M tris, 0.001% bromphenol blue,

pH 6.8), boiled for 10 minutes, and centrifuged at 10000 g for 10 more minutes. The obtained supernatant was applied onto the gel and separated in a running buffer system of 192 mM glycin, 0.1% SDS, and 25 mM tris, pH 8.3. The voltage was 100 V during the first 30 minutes, then it was increased to 150 V for the following 2 hours. The gels were stained in a solution of 0.1% coomassie brilliant blue, 50% methanol, and 10% acetic acid. For destaining they were put into 25% ethanol with 8% acetic acid until the background was clear. Then pictures were taken from the gels on ILFORD Pan F film (50 ASA = 18° DIN) with a yellow filter. From the pictures selected bands were scanned with a densitometer (pdi, model DNA 35) using the software Quantity One, One Dimensional Analyses, version 2.1.

2.2.4. Immunoblotting

The immunoblottings were accomplished according to BALDO & al. 1986, TOVEY & al. 1987, and TSANG & al. 1983. First the raw protein extracts were separated with SDS-PAGE as described above. Ensuing the gels were put onto nitrocellulose, that had been soaked in blotting buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol, pH 9.9). Gel and nitrocellulose were put into a trans blot cell (BIORAD) filled with blotting buffer. The electrophoretic transfer of the proteins from gel to nitrocellulose lasted 3 hours at a voltage of 30 V. Afterwards the nitrocellulose was saturated for 30 minutes in PBS-tween (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 0.2% tween 20, pH 7.5).

The antisera for the immuno staining had been risen in rabbits immunized with lichen protein extracts from 1800 m, 1000 m, or 750 m height above sea level. The antisera were diluted 1 : 1000 with PBS-tween and the blotted nitrocellulose was incubated in one of them respectively for 3–4 hours. Three short washings in PBS-tween followed, then the incubation of the nitrocellulose in a goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate (BIORAD), diluted 1 : 1000 with PBS-tween was performed for several hours. After three short washings in PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.2) the nitrocellulose was stained in a solution of 60 mg chlornaphthol in 20 ml methanol and 60 µl H_2O_2 in 100 ml PBS until the optimal banding was reached. The blots were transferred into PBS and pictures were taken on ILFORD Pan F film.

2.2.5. Two-Dimensional Gel Electrophoresis

The separation of the proteins in the first dimension was accomplished according to their charge as described by O'FARRELL 1975. In the second dimension their molecular weight was used for separation in SDS-PAGE.

2.2.5.1. Isoelectric Focussing (IEF)

Rod gels with a length of 75 mm and a diameter of 1 mm were casted from a solution of 5.5 g urea, 1.3 ml acrylamide stock (28,4% acrylamide, 1.6% bisacrylamide), 2.0 ml triton X-100, 1.7 ml bidistilled water, 0.60 ml ampholyte 5–7 (BIORAD), 0.12 ml ampholyte 3–10 (BIORAD), 5 µl TEMED, and 10 µl ammonium persulphate. On each of the rod gels 30–50 µl protein extract (as described in 2.2.2.2.) were applied and covered with 15 µl of overlay buffer (8.0 M urea, 1% ampholyte 7–9 (BIORAD), 5% triton X-100, 5% 2-mercaptoethanol). The electrophoretic run was performed with 20 mM NaOH as cathodic buffer and 10 mM glutamic acid as anodic buffer at 500 V

for 30 minutes followed by 1500 V for 2 hours and 30 minutes. After electrophoretic separation the rod gels were equilibrated in SDS sample buffer for 30 minutes and either used for the electrophoretic separation in the second dimension or stored frozen.

2.2.5.2. SDS-PAGE

In the second dimension protein separation was carried out by SDS-PAGE according to LAEMMLI 1970 similarly as described in 2.2.3.. The separation gel contained 12.3% acrylamide, 0.4% bisacrylamide, 0.2% SDS, 0.38 M tris, pH 8.8, 0.05% TEMED, and 0.033% ammonium persulphate. Its size was 8.0 cm × 6.0 cm × 0.1 cm. The ingredients of the stacking gel are given in 2.2.3. The equilibrated rod gel was put onto this stacking gel, and the electrophoretic run was accomplished in the same running buffer as in 2.2.3. at 100 V for 30 min and at 150 V for the next 75 minutes. The gels were stained in coomassie brilliant blue and destained (see 2.2.3.). These procedures were followed by the more sensitive silverstaining according to MERRIL & GOLDMAN 1984. Then pictures were taken from the gels on ILFORD Pan F film with a yellow filter.

3. Results

3.1. Comparison of the Protein Composition after Separation by SDS-PAGE

We prepared at least five parallel extractions to check the variation of the protein pattern in a population and to control the reproducibility of our techniques. It turned out that the intrapopulational variation was negligible in comparison to the interpopulational one. The manual casting of the gels and the separation in different electrophoretic runs resulted in some smaller variation in the banding pattern. Fig. 1 shows protein extracts from all the investigated habitats after SDS-PAGE. For a more detailed analysis specimens of the different altitudes are discussed in pairs. The samples from 50 m and from 300 m height above sea level show no significant differences. The band at 81 kD in the extracts from 50 m cannot be taken into consideration because it was not reproducible in all the protein extracts. It might be an artefact caused by epilichenic bacteria or not lichenized green algae. A first small and not quantitative difference appeared between the samples from 300 m and 750 m. The band at 73 kD is stronger in the 300 m extract. Comparing the 750 m and the 1000 m samples the band at 29 kD is richer in protein at 750 m. The same fact was observed for the band at 17 kD. The lichen samples from 1000 m and from 1100 m were identical to a large extent. Only at 29 kD a small increase in protein concentration appeared in outlines. The banding of the protein extracts from 1100 m and 1400 m were almost the same especially regarding that the protein extract from 1400 m was generally more concentrated than the 1100 m sample when loaded. Their high similarity is the more astonishing as the habitats are far away from each other with one in southern

Italy and the other in Austria. The protein composition from 1400 m and from 1500 m height above sea level indicated distinct differences. The bands at 29 kD, 48 kD, and 64 kD are less concentrated in the 1500 m sample. The bandings from 1500 m and 1800 m showed quantitative differences at 14 kD, 29 kD, 48 kD, and 73 kD. At 14 kD the protein concentration was higher in the 1800 m extracts, while the bands at 29 kD, 48 kD, and 73 kD were more prominent in the 1500 m lichens.

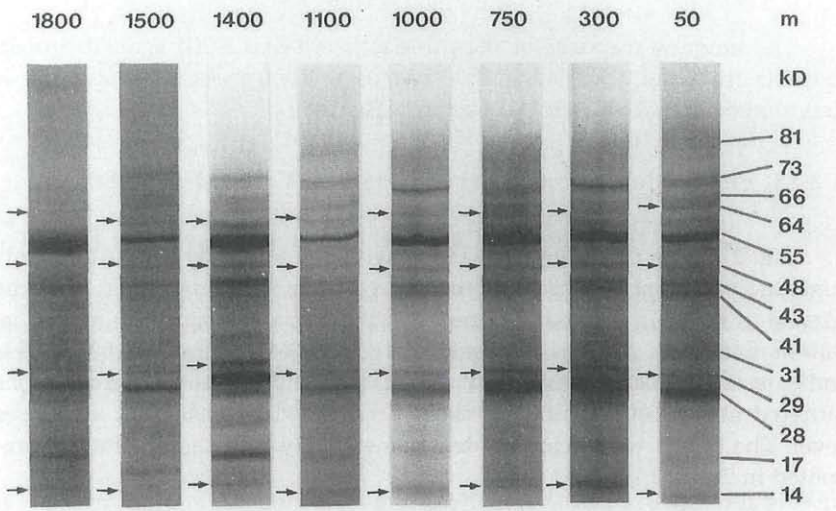


Fig. 1: Comparison of total protein extracts of *Pseudevernia furfuracea* var. *ceratea* after SDS-PAGE. The arrows indicate the most remarkable bands with variations in protein concentration. At 29 kD, 48 kD, and 64 kD a reduction of protein is connected with rising altitude; at 14 kD an increase in protein is seen.

All together a general trend towards a decrease of proteins with rising altitudes was indicated in the bands at 29 kD, 48 kD, and 64 kD. For the quantification of these differences with densitometrical scanning an extract from 1800 m and one from 750 m were chosen, because the habitats of these two samples were selected with special care regarding the similarity of their ecological conditions. The quantitative decrease of protein concentration with rising height above sea level was proved by densitometry for the bands at 29 kD, 48 kD, and 64 kD. The results are shown in Table 1.

Table 1

Comparison of the protein quantities of an extract from 1800 m with one from 750 m after SDS-PAGE

Band	R_f	Relative quantity in % 1800 m	750 m
64 kD	0.35	1.5	2.0
48 kD	0.47	1.9	2.8
29 kD	0.65	2.1	3.3

3.2. Comparison of the Protein Composition after Separation by Immunoblotting

The immune response of the three antisera (AB I–III) against protein extracts from the habitats at 1800 m (AB I), 1000 m (AB II), and 750 m height above sea level (AB III) was investigated.

3.2.1. Protein Composition after Immune Reaction with the Antiserum against 1800 m Lichen Protein

Fig. 2 shows the immune reaction with the antibody pool AB I. Only high molecular structures are recognized. The bandings look uniform. Merely at 66 kD a fine band appears in the extracts from 1000 m, 750 m, 300 m, and 50 m while it is absent at 1800 m, 1400 m, and 1100 m. This indicates that the different habitats can be divided into two groups: one situated above 1000 m and another one below 1000 m height above sea level. The bands were scanned densitometrically, and the results are presented in Table 2.

Table 2

Densitometric scanning of the band at 66 kD in the immunoblots shown in Fig. 2

Height above sea level in m	Relative quantity of the 66 kD band in%
1800	3.1
1400	3.0
1100	4.1
1000	7.0
750	5.1
300	5.5
50	6.3

3.2.2. Protein Composition after Immune Reaction with the Antiserum against 1000 m Lichen Protein

Fig. 3 shows the protein compositions with only minor variation after incubation with the antiserum AB II. Merely the band at 66 kD is different:

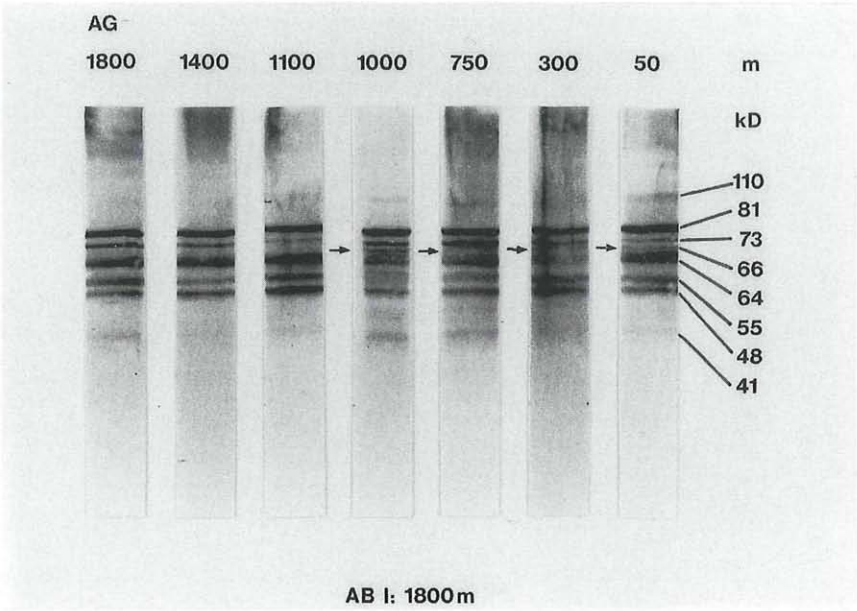


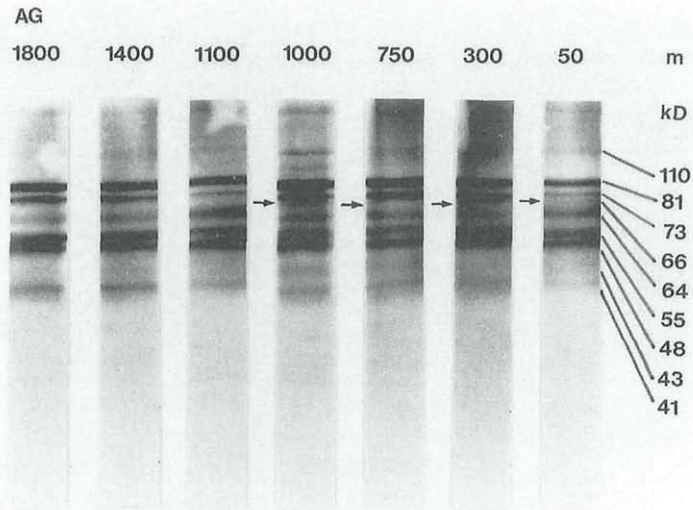
Fig. 2: Immunoblots of the total protein of *Pseudevernia furfuracea* var. *ceratea*. Protein extracts from different altitudes were used as antigens (AG). The antibodies came from the rabbit antiserum (AB I) against the lichen protein from 1800 m. At 66 kD a band appears in the blots with antigens from lichens at 1000 m, 750 m, 300 m, and 50 m, while it is missing in higher altitudes.

It is absent in habitats above 1000 m (1800 m, 1400 m, 1100 m) but present in samples from lower sites (1000 m, 750 m, 300 m, 50 m). The quantitative differences of this band at 66 kD are given in Table 3.

Table 3

Densitometric scanning of the band at 66 kD in the immunoblots shown in Fig. 3

Height above sea level in m	Relative quantity of the 66 kD band in%
1800	2.2
1400	2.3
1100	2.5
1000	5.2
750	3.2
300	4.6
50	4.6



AB II: 1000 m

Fig. 3: Immunoblots of the total protein of *Pseudevernia furfuracea* var. *ceratea*. The blots with lichen proteins from 1000 m, 750 m, 300 m, and 50 m as antigens (AG) show a band at 66 kD that is not recognized by the antiserum AB II (against lichen protein from 1000 m) in higher altitudes.

3.2.3. Protein Composition after Immune Reaction with the Antiserum against 750 m Lichen Protein

As shown in Fig. 4 the band at 66 kD is also recognized in the extracts from 1000 m, 750 m, 300 m, and 50 m. The antibodies used came from the antiserum AB III. At 1800 m, 1400 m, and 1100 m the band at 66 kD was missing as before in Fig. 2 (AB I) and Fig. 3 (AB II). The results of the densitometric scanning are listed in Table 4.

Table 4

Relative protein amounts of the band at 66 kD in the immunoblots shown in Fig. 4 measured by densitometry

Height above sea level in m	Relative quantity of the 66 kD band in%
1800	2.0
1400	3.0
1100	2.7
1000	4.5
750	4.2
300	4.0
50	5.5

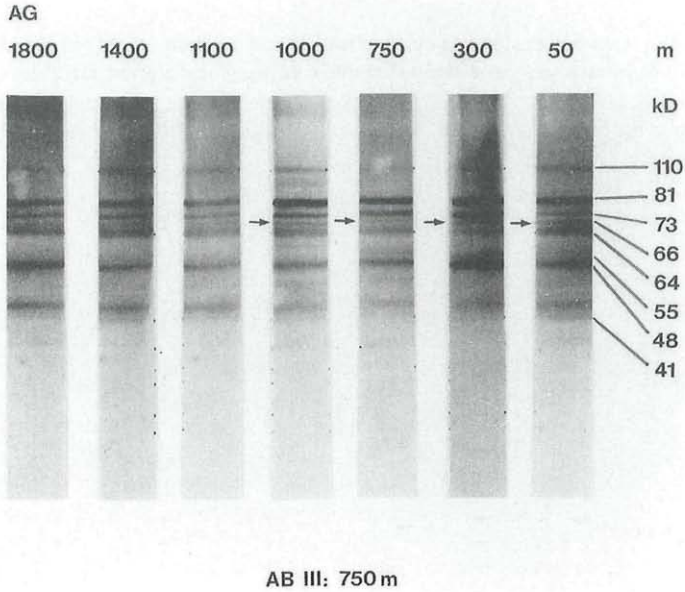


Fig. 4: Immunoblots of the total protein of *Pseudevernia furfuracea* var. *ceratea*. The antiserum AB III against lichen protein from 750 m bound to a band at 66 kD in the protein extracts from sites below 1000 m (AG: 1000 m, 750 m, 300 m, 50 m) but not above 1000 m.

3.3. Protein Composition after Separation by Two-Dimensional Gel Electrophoresis

Fig. 5 and 6 show – representative for all the other altitudes – the protein pattern of the lichen extracts from 1800 m and from 300 m after two-dimensional gel electrophoresis. For interpretation only the not overloaded parts of the gel were used. Therefore sectors with mainly high molecular weight proteins had to be omitted because of diffuse resolution.

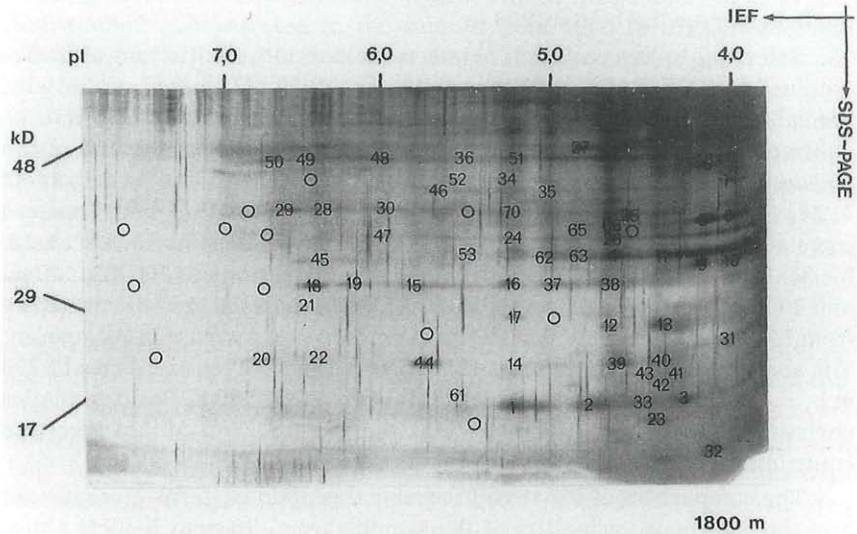
All together 72 spots per gel were evaluated, 27 of them were present at all the investigated altitudes, while 45 spots showed qualitative differences. The spots that were not found in all gels are listed in Table 5. From this it can be seen, that the missing of certain proteins at different altitudes varies. The spots in Table 5 were ordered to establish similar groups. It is remarkable that the spots 54, 55, 56, 57, 58, 59, 60, and 64 are all missing in the protein extracts from 1800 m, 1100 m, and 1000 m, i. e. the lichens growing at and above 1000 m. Nevertheless a classification of the lichen samples by habitats into two groups with one above and another one below 1000 m height above sea level can be given only with restraint: The sample from 1400 m takes an exceptional position but since it has many protein spots in common with the lichens at 1800 m, 1100 m, and

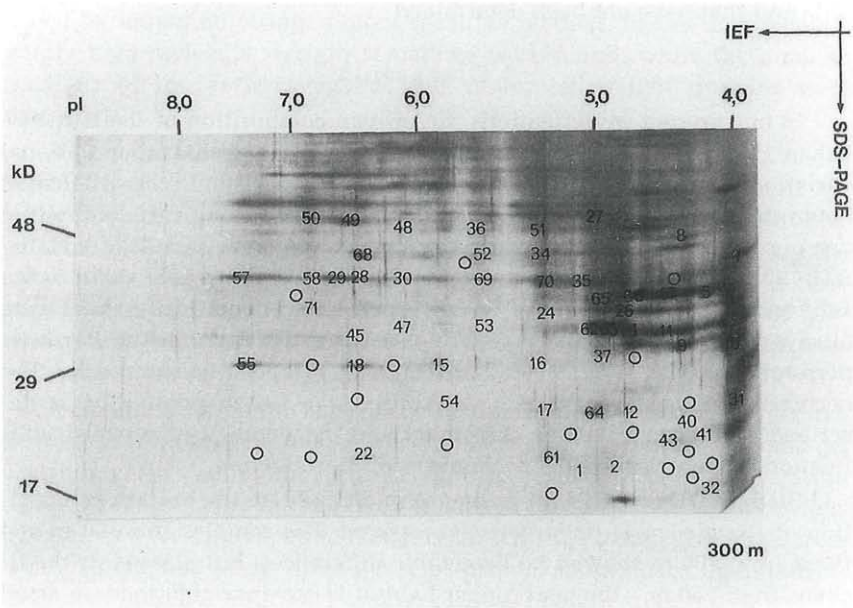
Table 5

List of the two-dimensional gel electrophoresis protein spots of *Pseudevernia furfuracea* var. *ceratea* qualitatively varying at different altitudes

Proteinspot	1800 m	1400 m	1100 m	1000 m	750 m	300 m	50 m
1	+	+	+	+	+	+	–
7	+	+	+	+	+	+	–
18	+	+	+	+	+	+	–
29	+	+	+	+	+	+	–
62	+	+	+	+	+	+	–
66	+	+	+	+	+	+	–
33	+	+	+	+	+	–	+
3	+	+	+	+	+	–	+
19	+	+	+	+	+	–	+
20	+	+	+	+	+	–	+
38	+	+	+	+	+	–	+
39	+	+	+	+	+	–	+
44	+	+	+	+	+	–	+
5	+	+	+	+	–	+	+
6	+	+	+	+	–	+	+
22	+	+	+	+	–	+	+
34	+	+	+	+	–	+	+
40	+	+	+	+	–	+	+
47	+	+	+	+	–	+	+
13	+	+	+	+	–	–	+
23	+	+	+	+	–	–	+
42	+	+	+	+	–	–	+
35	+	+	+	+	–	+	–
50	+	+	–	+	+	+	+
61	+	+	–	+	+	+	+
37	+	–	+	+	+	+	+
68	–	+	+	+	+	+	+
43	+	–	+	+	+	+	–
46	+	–	+	+	+	–	–
25	+	–	+	+	+	–	+
69	–	–	+	+	+	+	+
14	+	+	–	–	+	–	+
21	+	+	–	–	+	–	+
65	+	+	–	–	+	+	-
58	–	+	–	–	+	+	–
54	–	+	–	–	+	+	+
55	–	+	–	–	+	+	+
57	–	+	–	–	+	+	+
56	–	+	–	–	+	–	+
60	–	+	–	–	+	–	+
59	–	+	–	–	–	–	+
71	–	+	–	–	–	+	+
64	–	+	–	–	+	+	+
67	–	–	+	–	+	+	–
72	–	–	–	–	+	–	-

+ indicates the presence of a spot at a corresponding height above sea level, – stands for its missing.





Figs. 5 and 6: Two-dimensional gel electrophoresis of the total protein of *Pseudevernia furfuracea* var. *ceratea* from 1800 m and 300 m. The protein spots were numbered for easier identification. Circles indicate positions where proteins were detected at other heights above sea level but not in the present gel.

1000 m it seems to be more related to lichens from higher altitudes than to the lichens growing below 1000 m.

Referring to the variation between the habitats at different altitudes obtained by SDS-PAGE the zones at 48 kD and 29 kD were observed with special care. The SDS-PAGE differences at 64 kD were impossible to be analyzed more exactly by two-dimensional electrophoresis because of the bad resolution in this part of the gels. At 48 kD there are the protein spots 8, 36, 48, 49, 50, and 51. They show only small variation that does not correlate with the height above sea level. At 29 kD the spots 15, 16, 18, 19, 37, 38, 55, and 56 are found. Spot 55 and 56 are missing at 1800 m, 1100 m, and 1000 m. Spot 56 also does not exist at 300 m. In the gels with samples from 1400 m, 750 m, and 50 m this protein shows an increase in its amount. The absence of these proteins at 1800 m with their obvious presence at 750 m is in conformity with the result obtained by SDS-PAGE. Besides smaller variation of the other 29 kD proteins the spots 55 and 56 are therefore contributing a lot to the different bandings seen in SDS-PAGE.

The comparison of the two-dimensional protein patterns gives insight into the enormous variability of the samples from different heights above sea level. A multitude of proteins is expressed with different strength; the reason for this might lie in stressors that are not only depending on altitude and that have not been determined.

4. Discussion

In our present investigations the protein composition of the fruticose lichen *Pseudevernia furfuracea* var. *ceratea* showed an intrapopulational variation but this turned out as negligible in comparison to the differences between different populations. Different bandings among various thalli of one population are also described for *Cetraria arenaria* (FAHSELT & HAGEMAN 1983), *Umbilicaria mammulata* (HAGEMAN & FAHSELT 1984), and *Usnea subfloridana* (FAHSELT 1985). The intraspecific isoenzyme differences were always lower than the interspecific ones. Investigations on the *Parmelia perforata-complex* (FAHSELT & JANCEY 1977) showed the same results. The occurrence of some enzymatic variability in a lichen population is described by FAHSELT 1988 as customary and independent of sexual fructification or mere vegetative mechanisms of reproduction.

SDS-PAGE revealed an influence of altitude on the protein composition of *Pseudevernia furfuracea* var. *ceratea*. The samples from 50 m and those from 300 m showed no detectable differences, but already in the lichens from 750 m – the next higher habitat in our investigations – a small decrease in protein concentration was found at the band at 73 kD. Comparing the 750 m extracts and the ones from 1000 m a protein reduction was detected in the bands at 29 kD and 17 kD in the lichens from higher altitudes. The further elevation of height above sea level resulted in de-

creasing protein concentrations of several bands: the proteins at 29 kD are slighty more concentrated in the sample from 1000 m than in the only 100 m higher situated lichens from Italy. The next investigated altitude was 1400 m, where we could not detect a difference to the samples from 1100 m with SDS-PAGE. Nevertheless an altitude of 1500 m brought along differences to the just mentioned habitat at 1400 m: the protein bands at 29 kD, 48 kD, and 64 kD were again stronger at the lower sites. In comparison of the samples from 1500 m with the lichens from our highest habitat (1800 m) at timberline a decrease of the proteins with 29 kD, 48 kD, and 73 kD molecular weight was recognized at rising altitudes. However, the band at 14 kD is richer in protein at 1800 m than at 1500 m.

To make the influence of the altitude more clear a comparison of lichens from 1800 m and from 750 m was done. This bigger elevational difference aimed to reveal a possible creeping change, that might be overlooked in comparing only small elevational steps. With SDS-gels and with their densitometric scannings we could show that the protein bands with the molecular weights of 29 kD, 48 kD, and 64 kD were more concentrated at 750 m than at 1800 m. Thus a protein reduction exists in *Pseudevernia furfuracea* var. *ceratea* with rising altitudes.

Further informations about this protein reduction should be obtained by immunoblotting, a more sensitive method. In the immunoblots mainly high molecular weight structures were found, while the immune reactions of the antisera against low molecular weight proteins were only weak.

AB I – our antiserum against the raw protein extracts of the lichens from 1800 m – recognized besides other common proteins a band at 66 kD in the samples from 1000 m, 750 m, 300 m, and 50 m. At higher altitudes (1100 m, 1400 m, 1800 m) this band was not present. The comparison of the antisera AB II and AB III against the raw protein extracts of the lichens from 1000 m and from 750 m showed the same difference at the 66 kD-band. These were missing in lichens growing above 1000 m, while they were present in the lower situated lichens.

A classification of the habitats of *Pseudevernia furfuracea* var. *ceratea* in sites above and below 1000 m was also indicated by two-dimensional gel electrophoresis. While the protein spot patterns of the samples from 1800 m, 1400 m, 1100 m, and 1000 m looked quite similar, more obvious differences were discovered in comparison with the lichen groupings from 750 m, 300 m, and 50 m. Although some protein spots varied in all our investigated habitats in quality and quantity a classification in two groups at the 1000 m contour line seems to be justified, considering the entirety of all spots. This is more valid in regards to the multitude of parameters connected to the altitude such as light influx, UV radiation, temperature differences, precipitation, duration of the vegetation period, sun and shade

changes, et cetera. All of them affect the lichen physiology at very different cardinal points of metabolism.

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