Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation

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Abstract

Salivary proteins (SPs) of Schizaphis graminum, Acyrthosiphon pisum and Myzus persicae were studied after probing and feeding on different artificial diets. Salivary sheaths as well as apical lumps of saliva were found, presumably representing subsequently excreted saliva of different types. Phenoloxidase, pectinase and peroxidase activities were detected by staining the enzyme-converted products, thus confirming these enzyme activities found earlier by others. Proteinase and cellulase were not found. SPs in three major SDS–PAGE bands, at 154 and 66/69 kDa, were collected in fluid diets (soluble fraction) and as sheath material (solid fraction) attached to the membranes covering these diets. Proteins of both fractions presumably represented the enzymatic activities found, although this could not be proven. The lack of electrophoretic mobility of the undenaturated (isoelectrofocusing and PAGE) active proteins meant that they could not be separated, whereas the mobile denaturated (SDS–PAGE) proteins had lost their enzyme activity. Polyclonal antibodies, anti-SP154 and anti-SP66/69, both cross-reacted to most salivary proteins in Western blots. They also reacted to sheath material and to the principal salivary glands. For further studies of saliva some monoclonal antibodies were developed. The complexity of salivation and the relation of the results obtained to the behaviourally known secretion periods is discussed. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Salivary glands; Proteins; Immunolocalisation

1. Introduction

The aphid Schizaphis graminum (greenbug) is known as a serious pest for a variety of cereals, causing major economic damage. Stylet penetration, or probing, by S. graminum often causes chlorosis and sometimes necrosis in leaves of cereals (Ryan et al., 1990). This damage by the plant’s reactions to aphid probing develops in a time span of days. Presumably, the aphid saliva plays an important role in the plant’s chlorosis as the direct cell damage by the penetrating stylets through the plant tissue is only minute, at least initially, in spite of many intracellular punctures (Tjallingii and Hogen-Esch, 1993). At the level of sieve elements in the phloem, there is also no indication of serious damage. Though careful puncturing of the sieve element by a glass microelectrode evokes plugging of its sieve plate within minutes (Knoblauch and van Bel, 1998), an aphid is apparently able to puncture and tap sap from a sieve element for hours and days, continuously (Tjallingii, 1995). Possibly here also, the saliva injected before phloem ingestion may play an important role.

Electrical penetration graph (EPG) studies have shown (Tjallingii, 1988; Prado and Tjallingii, 1994; Martin et al., 1997; Tjallingii and Cherqui, 1999) that there are at least four phases of salivary secretion during plant penetration: (1) intercellular sheath secretion, (2) intracellular salivation into cells along the stylet path, (3) initial phloem salivation (into sieve elements), and (4) phloem feeding salivation (added to the ingested sap). Only sheath saliva is a gelling substance, the saliva of the other secretion phases is watery. Others have claimed the excretion of watery saliva (Miles, 1959) occurring in alternation with sheath secretion (McLean and Kinsey, 1965) or postulated that it could be inferred...
from detected enzyme activity (Urbanska et al., 1998). It remains unclear how these observations, all done with aphids probing into artificial diets covered by membranes, are related to probing into plants.

Diet-collected saliva, i.e. saliva obtained after excretion by aphids in Parafilm-covered fluid diets, has been analysed and proteins have been separated by electrophoresis (Miles, 1999). Baumann and Baumann (1995) showed that saliva of S. graminum contained three main proteins with homologue sequences in their N terminus, suggesting a structural and synthetic relationship. Miles and Harrewijn (1991), on the other hand, found proteins of different molecular weights in diet-collected saliva from the aphid Myzus persicae. The aphid species as well as the diet compositions used in these studies differed so that it remains unclear what caused these differences. Also, these studies provided no indication where and when these proteins are secreted in plants. Phenoloxidases were found in aphid saliva (Miles, 1985) and there were speculations about their detoxifying function in plant tissues (Leszcynsky and Dixon, 1990; Miles and Oertli, 1993). Urbanska et al. (1998) confirmed the phenoloxidase activity in the aphid’s saliva excretions. Pectinase activity has been detected elegantly by Ma et al. (1990) in salivary excretions of S. graminum. The need of pectinase for stylet penetration between cells has been postulated (McAllan and Adams, 1961; Campbell and Dreyer, 1990). However, its interference with the physiology of plants by the release of pectin fragments, known as potent elicitors, may be more important (Ma et al., 1998). Other enzyme activities in aphid saliva have been detected as well (Peng and Miles, 1988; Miles and Peng, 1989; Urbanska et al., 1998).

The injected saliva seems to play a crucial role in the prevention of the plant’s wound responses and damage but, on the other hand, it may act as an elicitor of plants reactions, resulting in damage during a later stage of the infestation. Whether the compatibility between plant and aphid species, the result of the evolutionary developments, really has these conflicting aspects forms the underlying question of this study. First, however, the role of the saliva should be shown and its composition during the various excretion periods must be established. The direct objective of this pilot study, therefore, was to try different diets for the collection of saliva, using different aphid species. For saliva analysis, assays for enzyme activity and techniques for protein separation were used to compare results with those obtained by others and to explore their technical prospects. We also wanted to initiate antibody experiments and to test them as a tool for future studies, such as characterisation of salivary components, tracing their origin from different salivary glands and their secretion in different plant tissues and cells. The use of some existing polyclonal and newly developed monoclonal antibodies can be seen as first attempts that should be extended further.

2. Materials and methods

2.1. Aphids and saliva collection

The three species of aphids were reared in the greenhouse at 20°C and a 16L:8D photoperiod. Myzus persicae (M1 clone, WAU, Wageningen) was reared on Brussels sprouts, Acrystosiphon pisum (green clone from INSA, Villeurbane, France; Dr Y. Rahbé) on broad beans, and Schizaphis graminum (clone from University of California, Davis, USA; Dr P. Baumann) on wheat. Up to 100 adults corresponding to 50 mg, were transferred from plants to a feeding chamber, a standing cylinder (PVC tube), 27 mm in diameter and 20 mm high, covered on top by a double layer of Parafilm™ with one of the diets in between (a “sachet”). Each sachet contained about 100 µl of diet. Aphids remained in the chamber overnight in an incubator at 20°C, illuminated from above with a constant yellow light (yellow foil filtered). Four types of diets were used (Table 1); (1) distilled water, (2) 15% sucrose, (3) 15% sucrose containing 100 mM serine, 100 mM methionine and 150 mM aspartic acid, and (4) an anti-clotting diet. The latter diet contained 50 mM citrate buffer, pH 6.2, and 10 mM EDTA and 120 mM NaCl to reduce clotting or gelling of salivary sheath material. Diets were microbial filtered and experiments were done in sterile conditions to avoid bacterial or fungal contamination.

The saliva collected after incubation of aphids on the diets can be distinguished as two fractions: (1) soluble saliva, which was in the fluid diets, collected after removal of the top membrane of the sachets, in addition to what was washed from the lower membrane with sample buffer (see below; an extra volume of about 20 µl per sachet), and (2) the sheath saliva, i.e. what remained attached to the lower membrane and was not suspended or dissolved in diets 1, 2 and 3. The collected saliva of about five sachets was pooled.

2.2. Sheath observations

After overnight incubation and removing the top membrane of the sachet, the solidified salivary sheaths remained attached to the lower membrane. Sheaths were stained by adding a drop of 0.01% Coomassie blue in 10% glycerol for 2 h. Then the stain was replaced by 10% glycerol, a cover-glass was put on top and sheaths were observed by light microscope. Also for sheath staining by antibodies and for enzyme detection, this procedure was followed using different staining methods (see below).
1 M NaCl. Cellulase activity would appear as a red spot on a white background.

2.4. SDS–PAGE and Western blotting

Analytical mini-SDS–PAGE was performed as described by Laemmli (1970). The soluble saliva fraction was centrifuged, and then the supernatant and the pellet were treated with a modified sample buffer. The sample buffer (pH 6.8) normally contained 100 mM Tris–HCl, 2% SDS, 20% glycerol and 0.01% bromophenol blue. In order to inhibit phenoloxidase activity and to dissolve the protein for better separation, the sample buffer was modified by adding PTU (phenylthiourea) and DTT (dithiothreitol), both 10 mM. The solid fraction of the saliva was collected by washing the protein mix from the Parafilm membrane with modified sample buffer. Iodoacetamide was added to 10 mM and the samples were boiled for 3 min in a water bath. The small amount of soluble proteins released by the aphids allowed no protein estimation (Bradford, 1976) without TCA precipitation. Proteins were applied to 4% stacking gel, and run in 10% electrophoretic gel at a current of 30 mA and silver stained. For calibration, standard proteins (Sigma kit) were used: myosin (205 kDa), β-galactosidase (114 kDa), phosphorylase b (97 kDa), bovine serum albumin (BSA; 67 kDa), ovalbumin (43 kDa) and bovine carbonic anhydrase (31 kDa) (as shown left of the lanes in Figs. 2 and 3).

Western blot testing by antisera followed the minigel electrophoresis. The salivary protein (SP) was transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a liquid transfer apparatus in a continuous buffer system at pH 11, containing 2.3 g/l of CAPS and 20% methanol, applying 0.8 mA/cm² for 1 h. Membranes were incubated overnight at 4°C in PBS pH 7.4 containing 0.1% Tween-20 and 1% BSA. After washing in PBS, the blots were incubated for 2 h with antisera, and diluted 1:2000 in PBS/0.1%BSA. Polyclonal anti-SP154 or anti-SP66/69 was used (against salivary proteins of 154 and 66/69 kDa of the S. graminum) donated by Dr P. Baumann, University of California, Davis; Baumann and Baumann, 1995). Anti-SP66/69 was active against SP66 and SP69. After three washes in PBS, the blots were incubated for 1 h in per-
oxidase-conjugated swine-anti-rabbit diluted to 1:2000 in PBS/0.1% BSA. After another step of washing in PBS, the blots were incubated in PBS containing 0.05% DAB and 0.01% H$_2$O$_2$ until a reddish brown colour appeared.

2.5. Isoelectrofocusing of saliva

Isoelectrofocusing (IEF) was performed under non-denaturing conditions. Proteins were electrofocused in a pH range 3.5 to 9.5 in precasted gel (Pharmacia) using 10 µl samples containing 10 mM of CHAPS. Electrode solutions for cathode and anode were 1 N NaOH and 20 mM H$_3$PO$_4$, respectively. A voltage of 1500 V was applied for 90 min. For pI calibration a mixture of 12 marker proteins was used (Pharmacia kit, containing: 1, amylo-glycosidase (3.5); 2, methyl red (3.75); 3, soya bean trypsin inhibitor (4.55); 4, β-lactoglobulin A (5.2); 5, bovine carbonic anhydrase (5.85); 6, human carbonic anhydrase B (6.55); 7, horse myoglobin acidic band (6.85); 8, horse myoglobin basic band (7.35); 9, lentil lectin-acidic band (8.15); 10, lentil lectin-middle band (8.45); 11, lentil lectin-basic band (8.65); 12, trypsinogen (9.35)). Gels were silver stained.

2.6. Histological procedures and immunodetection

Adult aphids with severed abdomens were submersed in Bouin–Holland sublimate, a fixative solution containing 12% formaldehyde (Vieillemaringe et al., 1984). Then specimens were dehydrated through a graded series of ethanol and amyl acetate and vacuum-embedded in Paraplast-Plus. Serial sections (10 µm) were cut and mounted on poly-l-lysine coated slides, de-paraffinised, blocked with 10% normal swine serum in PBS, and incubated for 2 h with the antisera SP154 or SP66/69 diluted 1:200. After 1:1 mixing with Freund’s incomplete adjuvant, the fractions were injected intraperitoneally into 15 mice, 1 mouse for each fraction. A second immunisation was given 4 weeks later, and 2 weeks later antiserum samples were collected and immunohistologically tested. Three mice were selected for hybridoma cell fusion experiments. They received a last booster injection 13 weeks after the first immunisation. After 3 days half of the spleen cells of these mice were separately fused with SP2/0 myeloma cells to result in hybridoma cell lines.

Antibody production was assessed by indirect immunolabelling, similar to the immunolocalisation for the polyclonal antibodies of S. graminum. This screening used salivary gland sections instead. Collected glands from A. pisum adults in 10-µm sections were mounted in poly-l-lysine coated wells of ELISA plates. After incubations with primary antiserum and secondary rabbit-anti-mouse peroxidase conjugate and staining, the sections could easily be screened.

3. Results

3.1. Salivary sheaths

Excreted salivary sheaths of three different aphid species in fluid diets (1, 2 and 3, Table 2) were clearly visible by light microscope after staining with Coomassie blue, indicating the protein nature of the sheath material. Sheaths remained attached to the penetrated lower membrane of the sachet. In addition to sheaths, distinct secretions at the end of the salivary sheaths were sometimes observed as stained swellings, referred to as “lumps” (Table 2, Fig. 1). In diet with anti-clotting buffer (diet 4, Table 2) fewer and smaller sheaths were found. Polyclonal antibodies anti-SP154 and anti-SP66/69 both stained the sheath material. The apical lumps of protein material, as observed in Coomassie blue stained sheaths (Fig. 1a), also reacted positively to the antisera when excreted by S. graminum (Fig. 1d).

3.2. Enzyme identification

Phenoloxidase was demonstrated in all diets that contained dopa. In fluid diets (diets 1, 2 and 3; Table 2) salivary sheaths of the three aphid species turned black.
Table 2
Detection of proteins and enzymatic activities of salivary excretions in different diets for three aphid species

<table>
<thead>
<tr>
<th>Diet</th>
<th>Substrate or stain</th>
<th>Aphids</th>
<th>Observations (LM)</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets 1, 2 and 3</td>
<td>Coomassie blue</td>
<td>S A M sheaths, halos</td>
<td>proteins</td>
<td></td>
</tr>
<tr>
<td>Diets 1, 2 and 3</td>
<td>Dopa</td>
<td>S A M sheaths, halos</td>
<td>phenoloxidase</td>
<td></td>
</tr>
<tr>
<td>Diet 4</td>
<td>Dopa</td>
<td>S A M sheaths</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>Diet 3+agarose</td>
<td>Dopa</td>
<td>S A M sheaths, halos</td>
<td>phenoloxidase</td>
<td></td>
</tr>
<tr>
<td>Diet 3+agarose</td>
<td>Pectin</td>
<td>S A M sheaths, halos</td>
<td>pectin methyl esterase</td>
<td></td>
</tr>
<tr>
<td>Diet 3+agarose</td>
<td>Pectin</td>
<td>S A M sheaths, halos</td>
<td>polygalacturonase</td>
<td></td>
</tr>
<tr>
<td>Diet 3</td>
<td>DAB</td>
<td>S A M sheaths</td>
<td>peroxidase</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Dopa, dihydroxyphenylalanine; DAB, diaminobenzidine; LM, light microscopy; S, Schizaphis graminum; A, Acrystisphion pisum; M, Myzus persicae.

Fig. 1. Salivary sheaths excreted in diets by Schizaphis graminum. Sheath and lump stained and observed after 24 h probing in a fluid or agarose diet: (a) sheath with lump in fluid diet 3 (without agarose) stained with Coomassie blue; (b) sheath and a concentration of dark material, which later will become a halo, in agarose diet 3 containing 0.1% dopa (dihydroxyphenylalanine); (c) agarose diet 3 containing 0.1% pectin, the Ruthenium red stain has disappeared around the sheath (light circular area) whereas it remained in the surrounding (pink, but grey in the photograph); (d) fluid diet 3 stained with anti-SP154 and horseradish peroxidase as secondary antibody. Bars 40 μm.

due to phenoloxidase activity after a few hours of aphid exposure. Also, halos were seen around some sheaths in fluid diets with dopa and they seemed to originate from the sheath’s apex. In agarose diets with dopa we observed halos as well as concentrations of dark material (Fig. 1b) around some (but not all) sheaths, which later became distinct halos. These halos and the dark material in the agarose diets are similar to the halos shown in fluid diets. All the visible staining indicated melanin due to phenoloxidase activity.

Pectinesterase and polygalacturonase activities were detected in diet 3 with agarose but not, or less obvious, in diets 1 and 2. Clear halos were found (Fig. 1c), which also seemed to originate from the sheath ends. With anti-clotting buffer, diet 4 (Table 1), no phenoloxidase or pectinase activity was observed.

A weak peroxidase activity was found in salivary sheaths of A. pisum and M. persicae, but not in salivary sheaths of S. graminum (Table 2). Thus, some detected enzyme activities seemed to depend on the diet composition and the aphid species. No activity could be detected in assays for cellulase and proteinase.

3.3. Salivary protein separations

SDS–PAGE of the soluble saliva fraction from S. graminum showed two main bands, SP154 and SP66/69 (Table 3). Depending on the diet, some differences were shown (Table 3, Fig. 2). When the time of silver staining was extended in diet 3 (containing sucrose and amino acids), some more bands appeared near the 43 kDa marker band (Fig. 2a; 40 and 30 kDa in Table 3), which were not present in diet 2 (without amino acids). In diet 4 (anti-clotting) we observed a major band at 154 kDa and a sharp band at 240 kDa, but neither at 66/69 kDa nor near 43 kDa were any proteins detected (Fig. 2b; Table 3). In the solid, salivary sheath fractions from diets 2 and 3, the SDS–PAGE showed only an SP66/69 band, no SP154. In the solid fraction of diet 3, as in the soluble fraction, additional bands were shown near 43 kDa, but not in diet 2 sheaths. Sheaths from the anti-clotting diet 4 showed a major band at 66/69 and a weak band at 154 kDa (Table 3).

In Western blots of the soluble fractions of all S. graminum probed diets, the polyclonal anti-SP154 reacted to SP154, to SP66/69 and (weakly) to proteins near 43 kDa (Fig. 3a). On the other hand, anti-SP66/69 reacted to the same proteins but not to SP154 and higher (Fig. 3b; Table 3). Sheaths fractions from diet 3 showed that anti-SP154 and anti-SP66/69 reacted only to SP66/69 in Western blots (Fig. 3c), as no bands higher than 69 kDa were present in the SDS–PAGE gels (Table 3). Proteins in neither soluble nor solid fractions from A. pisum or...
Table 3

Electrophoretic pattern of salivary fractions collected after exposure of *S. graminum* to different diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Saliva fraction</th>
<th>Electrophoretic pattern (kDa)</th>
<th>Polyclonal anti-SP154</th>
<th>Polyclonal anti-SP66-69</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet 2</td>
<td>soluble</td>
<td>154, 66/69</td>
<td>all</td>
<td>all, except 154</td>
</tr>
<tr>
<td></td>
<td>sheath</td>
<td>66/69</td>
<td>all</td>
<td>all</td>
</tr>
<tr>
<td>Diet 3</td>
<td>soluble</td>
<td>154, 66/69, 40, 30</td>
<td>all</td>
<td>all, except 154</td>
</tr>
<tr>
<td></td>
<td>sheath</td>
<td>66/69, 40, 30</td>
<td>all</td>
<td>all</td>
</tr>
<tr>
<td>Diet 4</td>
<td>soluble</td>
<td>240, 154</td>
<td>all</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>sheath</td>
<td>weak 154, 66/69</td>
<td>all</td>
<td>all, except 154</td>
</tr>
</tbody>
</table>

Fig. 2. Electrophoretic separation of salivary proteins. Proteins collected in two diets after 24 h exposure to *S. graminum* and submitted to SDS–PAGE under reducing conditions and silver stained. Pattern of soluble protein fractions in diet 3 (0.2 mg, lane a) and diet 4 (0.1 mg, lane b), and solid fraction (sheath material) in diet 3 (0.1 mg, lane c). Left, kDa values of calibration kit.

*M. persicae* reacted to the *S. graminum* anti-SP154 and anti-SP66/69.

Repeated attempts to use IEF for separation of components in soluble saliva, released during diet feeding, showed that the material applied in the samples did not migrate. Addition of zwitterionic detergent, 10 mM CHAPS, 6 M urea or 0.1% triton could not mobilise it. All loaded material remained in the deposit area as shown by silver staining. Immunoblots of the IEF gel showed that the same single spot was recognised by the anti-SP154 and the anti-SP66/69. Thus, classical separation procedures and subsequent biochemical techniques (Madhusudhan et al., 1994) could not be applied to the proteins without denaturation. Similarly, native PAGE electrophoresis was tried, without SDS pretreatment, at different pH values, with the same persistent lack of electrophoretic movement.

3.4. Immunolocalisation in aphids

Light microscopy of 10-µm sections of whole aphids (Fig. 4a) showed that anti-SP154 and SP66/69 (peroxidase conjugated detection) stained the posterior parts of the principal glands. The accessory salivary glands and the anterior cells of the principal glands were not stained. As peroxidase activity in the saliva (Miles and Peng, 1989; our own results above) or salivary
glands might give a false positive immunodetection (antibody detection was based on peroxidase conjugation) we incubated whole *S. graminum* sections without antibody treatment in DAB as controls. Staining anti-SP154 and anti-SP66/69 with fluorescein-anti-rabbit confirmed their specific binding to the posterior part of the principal salivary glands in *S. graminum* sections (Fig. 4d). No fluorescence was observed in accessory glands or other tissues. Salivary glands of *A. pisum* and *M. persicae* showed no reaction to the two *S. graminum* antibodies.

The six best monoclonal antibodies reacted to the accessory and principal salivary glands or only to the latter. Some cross reactivity to certain areas in the cerebral ganglia was observed as well. Tested on salivary sheaths, some showed positive reactions, others did not.

4. Discussion and conclusions

4.1. Saliva excretion and collection

The salivary sheaths and the apical lumps, excreted in diets, reacted similarly to protein staining by Coomassie blue and polyclonal antibodies. Phenoloxidase substrate (dopa), however, reacted to salivary sheaths but not to the clearly bordered lumps, resulting in more indistinct halos, which differed in appearance from the lumps. Therefore, the relation between lumps and halos is still unclear. However, we can conclude that although the sheath saliva and the lumps both contain salivary proteins, their enzyme activity is different. Phenoloxidase might be diffused from the lumps where it was not bound, as it was in the sheath material, or it might be secreted as “watery saliva”, independent of the lumps. Since we did not combine dopa diets with Coomassie blue, this remains unanswered at present.

The SP66/69 is the main protein excreted, as can be inferred from analytical SDS–PAGE and Western blots (Table 3). Immunolocalisation also indicated that these proteins are secreted by the proximal part of the principal salivary glands. The relation between SP66/69 and the activities of phenoloxidase, pectinase and peroxidase is not yet clear since the proteins lost their enzyme activity by denaturation for SDS–PAGE. The fact that anti-SP66/69 also stains the sheath lumps (Fig. 1d) does not indicate that SP66/69 cannot represent any enzyme activity, as we found very little specificity for the antibodies.
The halos seem to be the result of activity by diffused enzymes, proteins that were not bound (polymerised or not embedded in the gelling saliva) to the sheath, rather than the diffused enzyme products themselves. The pectinase halos, for example, reflect the absence of pectin (the substrate) converted by the enzymes, not the presence of the reaction product. Hence, the clear halos indicate the position of the enzymes themselves. Whether or not halos are caused by separate watery salivation or must be considered as the consequence of the protein lumps remains unclear. Both halos and lumps were not shown with all sheaths but halo absence could also be explained by a time lapse between sheath secretion and halo development. After 24 h exposure, only the sheaths produced early might have already developed halos, whereas near the later ones sufficient enzyme activity has not yet taken place. The intermediates, the observed dark concentrations (Fig. 1b), made it clear that there is a certain time needed for the halos to develop. Our findings do not seem to disagree with watery salivation occurring intermittently with or after sheath salivation, a suggestion made by McLean and Kinsey (1965) and Urbanska et al. (1998). Possibly this salivation is identical to one of the watery salivation periods identified in EPG studies (Tjallingii and Cherqui, 1999). Watery salivation occurs in plants, intermittently with sheath salivation, during short intracellular punctures along the stylet pathway and their corresponding waveforms have been observed in EPGs from diets as well (waveform pd; Tjallingii, 1985; Powell et al., 1995). However, these excretion periods are very short, only about 1 s, so that their contribution to the relatively large halo deposits seems questionable. Also, they would presumably not result in apical halos. Another watery salivation occurs in sieve elements after reaching the phloem, which is also responsible for the inoculation of persistent plant viruses (Gildow and Gray, 1993; Prado and Tjallingii, 1994). This waveform has also been shown by aphids on diets (waveform E1; Tjallingii, 1988). However, this saliva is thought to be secreted by accessory glands, at least partly, which may not be in agreement with the principal gland indications we found. Hence, further studies combining enzyme detection in diets with EPG recording are needed.

4.2. Identification of enzyme activity

Phenoloxidase activities in aphid saliva have also been detected by Miles and Peng (1989), Ma et al. (1990) and Urbanska et al. (1998). Catalytic reactions transform dopa to melanin in two steps. First, a hydroxylation of monophenol to o-diphenol and, subsequently, an oxidation of o-diphenols to o-quinones and their polymer, melanin (Cherqui et al., 1998). In insects, phenoloxidases are the key enzymes. They are involved in the tanning process of the cuticle and in the haemolymph for the defence reactions. In Homoptera (Miles, 1972) and the Heteroptera (Laurema et al., 1985) phenoloxidase seems to be present in the saliva and related glands. The phenoloxidase activity was observed in the salivary sheaths of aphids (Miles, 1972) and also in less structural deposits (halos) around the sheath material when excreted in agarose gels (Urbanska et al., 1998). Phenoloxidases probably oxidise the plant polyphenols to o-quinones. Plant penetration by aphids causes accumulation of polyphenols, especially catechin, presumably as a plant defence reaction (Peng and Miles, 1988; Miles, 1999). It remains unclear, however, what the benefit of this phenoloxidase excretion is for an aphid. Some suggest that the main function for the aphids is an in situ detoxification of phenols (Miles and Oertli, 1993; Leszczynski and Dixon, 1990). However, since there seems to be no ingestion of plant fluids during salivary sheath formation (pathway)—at least no substantial ingestion (Tjallingii, 1988)—this explanation is disputable.

Pectinases, pectinesterase and polygalacturonase have been suggested to be of major importance for intercellular stylet penetration, dissolving the pectin middle lamella (McAllan and Adams, 1961; Dreyer and Campbell, 1987; Campbell and Dreyer, 1990). However, transmission electron microscopy has shown that stylets predominantly follow the secondary cell walls, penetrating between the layers of cellulose fibres and not via the middle lamella (Tjallingii and Hogen-Esch, 1993). Pectinases may break down the pectin between the cellulose and hemicellulose fibres, but stylet penetration seems to go faster than the enzyme activity would allow. On the other hand, functional for stylet penetration or not, the pectinases will also cause pectin fragments, some of which might act as strong elicitors causing plant reactions (Ma et al., 1998).

4.3. Protein separation

Soluble saliva fractions from diets 2 and 3 showed two major bands of proteins in SDS–PAGE, SP154 and SP66/69, whereas in solid fractions only an SP66/69 band was present. Remarkably, the SP154 band was mostly absent (Table 3). All these proteins were recognised by anti-SP154 but, on the other hand, anti-SP66/69 did not recognise SP154. Baumann and Baumann (1995) identified the same S. graminum protein bands. They speculated that molecules of SP69 or SP66 form, or originate from, one molecule of SP154, or, on the basis of N terminus homology, that all three proteins have a common origin. Our data suggest that SP69 and SP66 form the salivary sheath but they are also excreted free into the diet (causing halos, Fig. 4a). As SP154 only occurs in the soluble fraction, it may be composed later from the SP66/69 molecules. However, the protein composition in diet 4 seems not to be in agreement with this, presum-
ably due to anti-clotting, although this is not completely understood.

The specificity of the polyclonal antibodies anti-SP154 and anti-SP66/69 to the saliva proteins appeared rather low, which may support the common origin. On the other hand, there were no cross-reactions observed to salivary proteins of other aphid species. Therefore, the low specificity within *S. graminum* proteins may indicate a common epitope for this aphid species as well.

4.4. Immunolocalisation

Aphids have two pairs of salivary glands, the principal and the accessory glands (Ponsen, 1972). Principal glands are divided into two parts, an anterior membranous part and a posterior glandular part. Polyclonal antibodies stained only the posterior cells of the principal salivary glands; neither the accessory glands nor the anterior cells of the principal glands were stained. Both peroxidase and fluorescence labels linked to the antibodies were unequivocal. This indicates that the principal glands secrete SP154, SP66/69 and other positively reacting proteins in our blots. SP69 and SP66 probably form the bulk proteins of the sheath material and are secreted by the posterior principal glands. The accessory glands might secrete the smaller proteins (near 43 kDa), as we suggested above. However, no antibody binding was found to the accessory glands: either there was no binding or it was too weak.

The results obtained demonstrated once more the complexity of saliva secretion by aphids. They also showed that understanding the functions of salivary secretions in the separate stages of plant penetration by the mouthparts requires a better knowledge of the secretion phases and how the two glands are involved. The role of the accessory glands, in particular, remained unknown in this study. With the diet collection method we presumably did not obtain all secretions of aphid salivary glands; neither the accessory glands nor the principal glands showed that understanding the functions of salivary proteins of other aphid species. Therefore, the low specificity within *S. graminum* proteins may indicate a common epitope for this aphid species as well.

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