Caterpillar regurgitant induces pore formation in plant membranes

Hinrich Lühring*, Van Dy Nguyen, Lilian Schmidt, Ursula S.R. Röse

Institute of Chemistry and Dynamics of the Geosphere, Phytosphere (ICG-3), Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany

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Abstract  Formation of channel-like pores in a plant membrane was induced within seconds after application of an aqueous solution containing regurgitant of the insect larvae Spodoptera littoralis. Gated pore currents recorded on the tonoplast of the Charophyte Chara corallina displayed conductances up to several hundred pS. A voltage-dependent gating reaction supports the assumption that pore-forming molecules have amphipathic properties. Regurgitant samples separated into masses smaller or larger than 3 kDa were evaluated by patch–clamp and mass spectroscopy. Fractions containing peptides larger than 3 kDa constituted pores of large conductances, peptides smaller than 3 kDa constituted pores of small conductances. Peptide-free eluates did not constitute conducting pores, indicating that pore-forming components in regurgitant are membrane-spanning oligopeptides.

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Keywords: Alamethicin; Caterpillar; Herbivory; Ion channel; Regurgitant; Chara

1. Introduction

Plant species like corn, cotton, lima beans and cultivated tobacco that are under attack by herbivorous insects are known to emit a variety of volatile compounds [1–4] that are partially de novo synthesised by the plant in response to herbivory [5]. Several of these plant-emitted volatiles function as signals in tritrophic interactions and may attract parasitoids or predators of the herbivores and therefore function as an indirect defence signal of the plant [2,6–8]. While caterpillar-damaged leaves of corn seedlings emit a complex blend of volatile compounds, mechanical wounding alone led to a far less complex blend. However, the application of caterpillar regurgitant to mechanically injured leaves resulted in a local and systemic emission of volatiles that was comparable to that emitted in response to herbivory [9–11] whereas regurgitant administered to intact leaf surfaces had no effect. Even when the mechanical damage to cotton plants was continued over several days, the systemically emitted amounts of several compounds were quantitatively lower than in those plants that were additionally treated with regurgitant [11]. It appears that a specific elicitor in the regurgitant of the caterpillar enhances the amount of several systemically released volatiles.

A compound isolated from the regurgitant of Spodoptera exigua larvae feeding on corn was identified as N-[17-hydroxylinolenoyl]-L-glutamine and was shown to induce the emission of herbivore-inducible volatiles when applied to mechanically wounded leaves. An application of this synthesised key component, named volicitin, elicited the production of volatiles [12]. Additional compounds in the oral secretion of Manduca sexta have been identified as N-linolenoyl-L-glutamine [13], and fatty acid–amino acid conjugates in herbivore oral secretions were shown to be necessary and sufficient for herbivore-specific plant responses and activation of the jasmonate signaling cascade [14]. Besides elicitors from herbivore regurgitant, fungal derived elicitors like alamethicin, an ion channel forming peptide from Trichoderma viride, may induce the emission of some of the volatiles emitted in response to herbivory. Alamethicin is well-known to produce pores in artificial membranes as well as in animal cell membranes, but has to date not been shown to form ion-channels in plant membranes. In Phaseolus lunatus, application of alamethicin induced the production of two homoterpenes and methyl salicylate whereas the complex blend of volatiles could not be observed [15].

From those results, a succession of primary events can be postulated: only intact cells in the neighbourhood of destroyed cells can produce metabolites; regurgitant must contain components (elicitors) triggering a cascade that leads to metabolite production; these components have to cross the plasma membrane to reach their adequate receptor molecules; to cross the membrane, a facilitator must mediate this transit; it appears reasonable that this facilitator molecule is also present in the caterpillar regurgitant. Assuming that the elicitor molecule is notably larger than an ion, the translocator of the elicitor should be a specific carrier-like molecule or simply an unspecific channel that may also transport ions. On artificial planar bilayer membranes, the existence of ion channel-like facilitators has recently been demonstrated [16]. Also elicitors with different functions like cellulolytic enzymes employed in cell wall digestion that had previously been presumed to act on plant cells via receptor binding sites proved to directly interact with membranes by formation of channel-like membrane pores [17].

To demonstrate the existence of an ion channel-like facilitator in caterpillar regurgitant and the pore formation of alamethicin in a plant membrane, we have chosen cytoplasmic droplets derived from internodal cells of the giant green alga Chara corallina that are delineated by the original tonoplast [18,19]. These droplets served as a model membrane system to evaluate permeation characteristics. The Chara tonoplast contains a highly specific maxi-K channel [19–21], a Ca2+-activated Cl− channel [22], and, as H+ pumps, a H+-ATPase as...
well as a H+-PPase [23–26]. The H+ pumps fuelled by ATP and pyrophosphate hydrolysation, respectively, are silent in the absence of their substrates and maxi-K and Cl− channel can be blocked by Cs+ ions or by withdrawal of Ca2+ ions, respectively. Thus, the Chara tonoplast provides a simple but adequate membrane to explore a possible new formation of membrane pores by exogenously applied caterpillar regurgitant and alamethicin.

2. Materials and methods

2.1. Collection of regurgitant of Spodoptera littoralis

Third instar larvae of S. littoralis (Lepidoptera: Noctuidae), reared on bean diet (shredded beans with vitamins) were placed in a container and fed on cotton leaves for 2–3 days before regurgitant was collected. Regurgitation of oral secretion was induced by holding the caterpillar in the head region with a pair of light-weight forceps. Oral secretion was collected with two 100-μl pipettes inserted in a vial through a septum. One pipette was connected to a low vacuum while the other pipette tip was held in front of the mouth part of the caterpillar. About

![Image](image-url)

**Fig. 1.** Alamethicin-induced conductances in the tonoplast of Chara corallina. In a cell-attached configuration, formation of a high-resistance seal and the absence of any current fluctuation at positive pipette voltages could be reconfirmed until alamethicin was inserting into the membrane. At negative voltages, current through the native maxi-K channel could be observed from the beginning. (A) Current fluctuations at +40 mV clamp voltage. (B) Voltage-dependency of alamethicin-mediated current. Currents of subsequent amplitude levels emerging in activity bursts were plotted versus clamp voltage. Lines connect the observed current levels that presumably belong to one conductance state. Conductances are summarised in Table 1. Experimental conditions – pipette and bath contained 120 mM CsCl, 5 mM Tris/HCl, pH 7.5; pipette additionally 11 μM alamethicin, 1 kHz Bessel low-pass, 50 μs/sample, Rpip 17 MΩ.

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<th>Lipid bilayer</th>
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<th>Chara corallina tonoplast</th>
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<td><strong>Results</strong></td>
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Results from C. corallina tonoplast recordings are compared to data collected from [29] and tabulated for comparison. Matching data are aligned, blank positions are meaningless.
5–10 µl of oral secretion could be collected per larva. The crude oral secretion was stored in the freezer at −80 °C until a sufficient amount was collected.

2.2. Electrophysiology

*C. corallina* was cultivated in large plastic buckets at 23 °C under a 12:12 h light:dark regime. Internodal cells of about 10 cm length and 0.8 mm diameter were isolated from adjacent cells and wilted to turgor loss. To prepare cytoplasmic droplets, one cell end submersed in K+ solution was cut and the cytosol gently squeezed out. Cytoplasmic droplets that formed readily in the bathing solution were picked up with a 20 µl pipette and transferred to the experimental chamber. Those droplets surrounded by the tonoplast were accessible by patch pipettes without further treatment. The solution for droplet preparation and the bathing solution in the experimental chamber contained 120 mM KCl and 5 mM Tris adjusted to pH 7.5. The patch pipettes were filled with solutions containing 120 mM CsCl, 5 mM Tris adjusted to pH 7.5, and additionally the substance under examination.

![Fig. 2. Current fluctuations occurring after spontaneous insertion of Spodoptera regurgitant components in the presence of ethanol contained in the aqueous pipette solution at five different clamp voltages. Clamp voltages are given left-hand side of the traces; closed levels are indicated right-hand side by a short line. Experimental conditions – bathing solution contained 120 mM KCl, 5 mM Tris, pH 7.5; pipette solution: 120 mM CsCl, 5 mM Tris, pH 7.5, 12.6 mg/ml regurgitant, 0.1% EtOH; *R*<sub>pip</sub> 5.8 MΩ, 1 kHz Bessel low-pass, inside-out configuration.](image)

In cell-attached an inside-out configurations, Cs+ in the patch pipette did not admit inwardly directed current fluctuations across

![Fig. 3. Current fluctuations occurring after spontaneous insertion of Spodoptera regurgitant components contained in the aqueous pipette solution in the absence of ethanol at three different clamp voltages. Clamp voltages are given on the left-hand side of the traces; closed levels are indicated on the right-hand side by a short line. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris, pH 7.5; pipette solution: 120 mM CsCl, 5 mM Tris, pH 7.5, 5 µl/ml regurgitant; *R*<sub>pip</sub> 15 MΩ, 1 kHz Bessel low-pass, cell-attached.](image)
intrinsic maxi-K channels at positive pipette voltages. Availability of K⁺ ions in the bathing solution allowed outward fluctuations at negative pipette voltages, thus providing information on the actual state of the membrane patch. Patch-clamp data were recorded and digitised with a MultiClamp 700A amplifier and Digidata 1322A acquisition hardware (both by Axon Instruments, Union City, CA).

Alamethicin (Fluka, Buchs CH) was presolved in ethanol and diluted to a final concentration of 11 μM in the aqueous pipette solution with less than 1% v/v ethanol.

Caterpillar regurgitant was added to the pipette solution containing less than 1% v/v ethanol to give final concentrations of 8–13 mg ml⁻¹ (depending on the collected sample size).

For a crude separation of molecular masses above 3 kDa and below 3 kDa, regurgitant was diluted into 120 mM CsCl to a final concentration of 11.7 mg ml⁻¹ and filtered through a 0.2 μm membrane filter (DynaGard, Spectrum Laboratories, Rancho Dominguez, CA) to remove undissolved particles for patch-clamp experiments. Subsequently, samples were separated according to their molecular weight above and below 3 kDa by filtering through a Microcon cell (YM-3, Amicon, Millipore, Billerica, MA) by centrifugation at 14000 × g for 30 min. Both fractions, >3 kDa and ≤3 kDa, were analysed electrophysiologically and by mass spectrometry. To determine whether the pore forming compound is a peptide, regurgitant was further concentrated by ZipTip pipette (Millipore, Billerica, MA). The peptide-free eluate from the ZipTip was tested for pore forming activity by patch-clamp and analysed by mass spectrometry. For subsequent mass spectrometry of peptides, peptides were eluted from the ZipTip with 0.05% trifluoro acetic acid and 50% acetonitrile.

2.3. Mass spectroscopy

Mass spectra were acquired with a MALDI-TOF spectrometer (Bruker Daltonik Biflex III, Bremen D), equipped with a multiprobe inlet and N₂ laser (337 nm, 3 ns pulse width). For the analysis of proteins, the linear-mode technique with an acceleration voltage of 19 kV and the reflectron voltage of 15.1 kV was applied. An ethanolic (<1% v/v) solution containing 117 mM CsCl, 4.65 mM Tris/HCl and 2.55 μM alamethicin was used for analysis. This solution was 1:100 diluted with a 0.1% aqueous solution of trifluoroacetic acid, and 5 μl thereof mixed with 5 μl of the matrix solution that was prepared as a 2:1 mixture of 0.1% trifluoroacetic acid and acetonitrile. CHCA (α-cyano-4-hydroxycinnamic acid) was added to a concentration of 20% w/v, and 1 μl of this final solution deposited on the Bruker Scout 26 target (dried droplet method). Mass spectra were acquired with delayed extraction of 200 ns. Angiotensin II (m/z 1047.2 [M+H]⁺ avg.), ACTH (adrenocorticotropic hormone fraction 18–39, m/z 2466.73 [M+H]⁺ avg.), and cytochrome c (m/z 12361.09 [M+H]⁺ avg.) were used as standards for calibration of the spectra, supported by software XTOF version 5.1.0 (Bruker Daltonics), as described elsewhere[27].

Of Spodoptera regurgitant (47.66 mg stocked in 1 ml of 120 mM NaCl), 200 μl were desalted and concentrated to 5 μl using a C₁₈ Millipore ZipTip pipette (Millipore) before analysis by mass spectrometry. Those pipettes are applicable for peptides and proteins of low molecular weight up to 50 kDa. Of the concentrated sample, mixed 1:1 with matrix solution, 1 μl was deposited on the target. For calibration of the spectra, the same standards were used as mentioned above. Size fractionated regurgitant samples and peptide-free and peptide-enriched samples were prepared as described above and analysed with the same method.

Fig. 4. Spodoptera regurgitant components forming high-conductance pores after spontaneous insertion into Chara tonoplast. Clamp voltages are given on the left-hand side of the traces; closed levels are indicated on the right-hand side. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris/HCl, pH 7.5, pipette solution: 120 mM CsCl, 5 mM Tris/HCl, pH 7.5, 12.6 mg/ml regurgitant, 0.1% v/v ethanol; R_{pip}: 5.2 MΩ, 20 kHz Bessel low-pass, inside-out.
3. Results and discussion

3.1. Alamethicin-induced pores in a plant membrane

To investigate the ability of alamethicin to induce pore formation in a plant membrane of Chara, alamethicin was applied to the Chara tonoplast at a concentration of 11 μM as a component of the patch pipette filling solution. Within about 30 s of administration of alamethicin in a cell-attached configuration, the first Cs⁺-conducting pores could be recorded (Fig. 1A). The different conductances of an alamethicin channel were obtained by measuring current levels at varying clamp voltages and plotting the current–voltage relationship for the detected conductant states (Fig. 1B). Alamethicin channels formed in the Chara tonoplast displayed conductance levels similar to those reported from artificial planar lipid bilayers, and frog or rat muscle membranes [28] showing that a spontaneous incorporation of alamethicin into the plant membrane may facilitate the intrusion of otherwise impermeant substances (Table 1). Conducting pores formed after administration of alamethicin at both membrane faces, the vacuolar as well as the cytosolic face, depending on the polarity of the applied voltage. Only a positive potential at the cis-side (locus of alamethicin application) compared to a more negative potential at the trans-side of the membrane, led to the formation of conducting pores. This is in accordance with observations on tobacco suspension cells where the tonoplast remains impermeable for low-molecular-mass molecules in response to alamethicin treatment [29]. The transitions between conductance levels are obviously faster than those reported for alamethicin pores in lipid bilayers. One reason may be that the commercially obtained alamethicin is a mixture of alamethicin analogues, wherein the Rf-30 component, the so-called major component, is contained by at least 50%. In lipid bilayers, functional pores formed by different alamethicin classes, like the Rf-50 type or mixtures of them, exhibit marked variances in dwell times for their conductance levels. The mean life time of the Rf-50 type of alamethicin, e.g., is by a factor of 5–10 lower than that of the Rf-30 type [30–32].

The oligopeptide alamethicin, well-known to produce pores in artificial and in animal cell membranes, and to elicit the production of volatile compounds in plant cells [15] is shown here to form conductive pores depending on the polarity of the applied voltage in plasma membranes of Charophytes, the class of green algae which is considered to be the immediate predecessor of land plants [33]. Chara qualifies as an excellent membrane model system, particularly seen in combination with its adequacy to serve as a heterologous expression system for, e.g., ion channels of mammal tissues provenience like acetylcholine receptor [34], 5-HT₃ serotonin receptor and CX32 gap junction protein (Lühring, unpublished data).

3.2. Caterpillar regurgitant contains a pore-forming compound

To explore the ability of caterpillar regurgitant to induce the formation of pores in plant cell membranes, patch pipettes were filled with an aqueous solution containing CsCl, Tris buffer and Spodoptera regurgitant. Setting the clamp voltage of the pipette in a cell-attached configuration to positive values of about +50 mV, the incorporation of pore-forming molecules could be detected by the induction of current fluctuations. With a positive pipette voltage, Cs⁺ prevents a current through the tonoplast-resident maxi-K channel and the observed current therefore had to be ascribed to an alien conduc-

tance induced by one or more compounds in the regurgitant. At negative pipette voltage, current fluctuations through the native maxi-K channel proved the integrity of the membrane. After patch excision into the inside-out configuration, current bursts were recorded at various clamp voltages (Fig. 2). Regurgitant-induced pores appear to be unspecific at least to monovalent cations, because changes in current amplitudes and also in reversal voltages could not be observed (results not shown). It is noteworthy that the presence of ethanol in the pipette solution (<1%, v/v), increased the life-time of current bursts of regurgitant-induced channels significantly (Fig. 2), whereas current fluctuations through the membrane-integrated regurgitant components in the absence of ethanol were rather short-lived (Fig. 3). Components in Spodoptera regurgitant may constitute pores of unusual long open times and very high conductance (Fig. 4). The long-lived open state of those high-conductance pores is extremely noisy and more than 10-fold that of the open state observed for low-conductance pores (Fig. 2), whereas the closed states of low- and high-conductance pores display a similar noise. Despite unusual great noise, current fluctuations could be recognised as single-channel events. Current fluctuations, recorded at a high sampling rate (100 kHz) were conditioned with an anti-aliasing filter set to 20 kHz corner frequency in order to prevent loss of information. These current fluctuations suggest that the regurgitant-induced membrane pores are not uniform, but rather that their formation should be very complex. This could be due to a changing number of components forming the transmembranal structure or perhaps to a single unit that may vary its configuration over a wide range. Application of volicitin, a compound isolated from caterpillar regurgitant that induces volatile emissions in plants [12], formed pores that exhibited a similar noisy current after reconstitution in artificial bilayers.

![Fig. 5. Current–voltage relationships of Spodoptera regurgitant-induced membrane conductances. Experimental conditions – bathing solution: 120 mM KCl, 5 mM Tris/HCl, pH 7.5, pipette solution: 120 mM CsCl, 5 mM Tris/HCl, pH 7.5, 12.6 mg/ml regurgitant, 0.1% ethanol, 2 kHz Bessel low-pass, inside-out.](image-url)
[16] as we observed for raw caterpillar regurgitant in plant membranes. However, in artificial bilayers, raw caterpillar regurgitant induced almost noise-free signals [16].

To detect and ascertain various conductance levels by means of amplitude histograms, whole data files were partitioned into time-limited fractions, rather than using the entire 1-min recording. Peak values of fractionated amplitude histograms, which were generated from recordings with apparently one conductant level, allowed constructing current–voltage relationships (Fig. 5). Here, conductances were determined to be

Fig. 6. Distributions of molecular masses of peptides A: smaller than 3 kDa (A) and larger than 3 kDa (B), from *Spodoptera* regurgitant dissolved in 120 mM NaCl and analysed by linear-mode MALDI-TOF MS. For comparison with the sample, calibration standards were deposited on the same target. Mass spectra of 234 μg each of *Spodoptera* regurgitant were generated in the presence of less than 30 nmol NaCl. The insets display electrical conductances typically found after spontaneous insertion of regurgitant compounds into *Chara* tonoplast. Conductances correspond to their fraction of molecular masses. Current traces recorded at +100 mV, cell-attached configuration, $R_{\text{pip}}$ 5 MΩ (A) and +140 mV, inside-out configuration, $R_{\text{pip}}$ 15 MΩ (B), low-pass filtered at 1 kHz.
about 167 pS and 517 pS, respectively. Obviously, there are even higher conductances which, however, could not be assigned to single pores. Size fractionation of regurgitant revealed that compounds in the regurgitant of molecular size below 3 kDa induced only formation of small pores with approximately 50 pS (Fig. 6A) in patch-clamp experiments, whereas compounds with a molecular size of more than 3 kDa induced the formation of large pores (ca. 600 pS, Fig. 6B). However, small amounts of low molecular mass compounds could not be excluded from the ≥ 3 kDa fraction. We

Fig. 7. Mass spectra after mass separation of Spodoptera regurgitant solutions from which peptides had been specifically removed by ZipTip treatment: (A) ZipTip-treated fraction smaller than 3 kDa. (B) ZipTip-treated fraction larger than 3 kDa. Spodoptera regurgitant had been dissolved in 120 mM NaCl, spectra were generated by linear-mode MALDI-TOF MS. For comparison with the sample, calibration standards were deposited on the same target. Less than 30 nmol NaCl were present in samples which contained 234 µg of Spodoptera regurgitant each. The insets display patch-clamp recordings on Chara tonoplast at the respective condition. Upper traces: no current events at peptide-free conditions, lower traces: membrane integrity shown by maxi-K channel activity at negative clamp voltage (low-pass filtered, 1 kHz).
can therefore conclude that compounds with masses above 3 kDa are necessary for the formation of large pores, either in combination with small amounts of lower molecular size compounds or by itself. Regurgitant filtered to specifically remove any peptides did not induce any formation of conducting pores in the plant membrane (Fig. 7A and B), although membrane patches were intact as shown by the activity of maxi-K channels at negative pipette voltage. These results strongly suggest that the compounds responsible for the formation of pores are peptides.

Frequently, open states could not be assigned to the appearance of a second (or third) pore, or reliably be diagnosed to be a subconductant state of the previously observed pore (Fig. 4, +100 mV trace). Nevertheless, kinetics of channel gating could be analysed in some recordings showing single events. For three recordings on one membrane patch mean open and closed life-times could be determined (Fig. 8A–F). Both, closed state as well as open state decayed in two reaction steps. The two open states were clearly distinguishable by their mean life times of about 1 ms ($O_1$) and 15 ms ($O_2$). Similarly, the closed states decayed with 1 ms ($C_1$) and 15 ms ($C_2$), respectively. Both rapidly decaying states, $O_1$ and $C_1$, were voltage-independent, at least over the range of 200–120 mV pipette voltage, whereas the slowly decaying states, $O_2$ and $C_2$, appeared to be voltage sensitive when stepping the pipette voltage from 160 to 120 mV. While the time constant of the long-lived open state $O_2$ was reduced from 15 ms to about 3 ms, the time constant of the long-lived closed state $C_2$ increased concomitantly from about 15 to almost 40 ms at the same voltage step. The simultaneous change of both time constants suggests a direct linkage in the gating reaction between $O_2$ and $C_2$. The time constants of $O_1$, $O_2$ and $C_1$, belong to transitions during burst activity. Since the open state may be abandoned via two different pathways, where only one of those is voltage-sensitive, the

$C_1 \rightarrow O_1 \rightarrow O_2 \rightarrow C_2$

Scheme 1.
of peptides contained in caterpillar regurgitant were detected, containing small amounts of the smaller fragments (Fig. 6A and B). Analysis of the eluate from the ZipTip-treated fractions revealed that none of them contained peptides (Fig. 8).

3.3. Mass spectroscopy

To analyse the chemical composition of our alamethicin and to carry out a first analysis of peptides in caterpillar regurgitant that may contribute to pore formation MALDI-TOF mass spectroscopy was employed. Spectrum analysis of alamethicin shows a peak with a m/z value of 2098.43 Da, which corresponds to the mean values of the alamethicin–Cs complex [alamethicin + Cs]⁺, comprising 132.9 Da for Cs and 1964.0 Da for the peptide. Analysis of regurgitant in fractionated samples of 234 µg of Spodoptera regurgitant shows 12 peptides, greater than 1 kDa each (Fig. 6). Surprisingly, it contained a peptide which exhibits a mass close to that of alamethicin, namely 1987.0 [peptide + Na]⁺, comprising 23 Da for Na and 1964.0 Da for the peptide. Analysis of regurgitant in fractionated samples of 3 kDa showed only formation of small pores, while in Schemes 2 and 3, as an alternative, C₂ may be attained from all other states C₁, O₁ or O₂. Transitions between O₁ and O₂, and C₁ and C₂ are permitted.

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Scheme 3.

3.3. Mass spectroscopy

To analyse the chemical composition of our alamethicin and to carry out a first analysis of peptides in caterpillar regurgitant that may contribute to pore formation MALDI-TOF mass spectroscopy was employed. Spectrum analysis of alamethicin shows a peak with a m/z value of 2098.43 Da, which corresponds to the mean values of the alamethicin–Cs complex [alamethicin + Cs]⁺, comprising 132.9 Da for Cs and 1964.0 Da for the peptide. Analysis of regurgitant in fractionated samples of 3 kDa showed only formation of small pores, while in Schemes 2 and 3, as an alternative, C₂ may be attained from all other states C₁, O₁ or O₂. Transitions between O₁ and O₂, and C₁ and C₂ are permitted.

Scheme 2.

Scheme 3.