Chapter 3

TRYPSIN EFFECT ON WATER SPLITTING SITE AND ITS RELATION WITH ELECTRON TRANSPORT IN NORMAL AND SALT-WASHED PHOTOSYSTEM II PREPARATION

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The oxygen evolving complex of photosystem II in higher plants involves the participation of three polypeptides with Mr of 33, 23-24 and 16-18 kD (Åkerlund and Jansson, 1981; Yamamoto et al., 1981; Åkerlund et al., 1982; Kuwabara and Murata, 1983; Miyao and Murata, 1983; Wensink et al., 1984; Ghanotakis et al., 1984a). These polypeptides are located on the inner surface of the thylakoid membrane (see review by Renger and Govindjee, 1985). Manganese (associated with 34 and 33 kD protein) is known to be essential in oxygen evolution Bishop, 1983; Miyao and Murata, 1984). It is known that salt-washing, treatment with Tris as well as with NH₂OH inhibit the oxygen evolving activity, though the degree of inhibition in each case is different (Murata et al., 1983). Also the quantum of released polypeptides is different (Yamamoto and Nishimura, 1983; Babcock et al., 1984; Yocum et al., 1984). Inside out thylakoid membranes have been used to investigate the role of these proteins (Jansson et al., 1979). Their purification, physico-chemical characteristics and contribution to oxygen evolution (Kuwabara and Murata, 1982; Jansson, 1984) have been studied. Ca²⁺ and Cl⁻ are required for optimum oxygen evolution. It has been proposed that 23 kD protein is responsible for trapping of Ca²⁺ and reduction in requirement for Cl⁻ (Andersson et al., 1984; Miyao and Murata, 1985). In PS II complex depleted of 23 kD protein, Ca²⁺ can reconstitute high rates of oxygen evolution (Ghanotakis et al., 1984b). Ca²⁺ is released from the functional site during illumination (Dekker et al., 1984; Murata and Miyao, 1987). Based on mild trypsinization of inside-out thylakoid membranes it was suggested that the electron transport activity may be affected by another protein exposed to the inner side of the thylakoid membrane besides the 18, 24 and 33 kD proteins (Renger et al., 1984). In PS II particles prepared with Triton X-100 (Ghanotakis et al., 1984a), trypsin is accessible to the acceptor as well as the donor side since this preparation consists of unsealed membrane fragments as shown by freeze fracture electron microscopic studies (Dunahay et al., 1984). Polypeptides on either side of the membrane can be digested by trypsin. Removal of the 18 and 24 kD proteins by salt-washing is likely to expose the lipid protein matrix of the secondary donor D₁. This protein plausibly provides a link between the Mn binding 34 kD polypeptide and the reaction centre complex. It seems after salt-washing certain intrinsic sites are likely to get exposed to trypsin action.

Volker et al. (1985) have analysed the pH dependence of the functional activities of trypsin treated PS II particles, Mn abundance and peptide pattern.
Our aim was to study the effect of proteolytic digestion on normal and salt-washed PS II particles with trypsin/Chl = 0.0001 to 1. We have attempted to correlate the structural modifications with the functional properties after trypsin treatment. We have also investigated the role of Ca\(^{2+}\) and NH\(_2\)OH in trypsin digested PS II particles.

2. Materials and Methods

The oxygen evolving PS II particles were isolated from spinach using the detergent Triton X-100 according to BBY method (Ghanotakis et al., 1984a). The preparation was stored at -80°C in 20 mM Mes-NaOH, pH 6, containing 0.4 M sucrose, 15 mM NaCl and 5 mM MgCl\(_2\).

To obtain salt-washed PS II, the stored sample was centrifuged at 40,000 x g for 30 min and the pellet was suspended in 20 mM Mes-NaOH, pH 6.0 containing 2 M NaCl and 5 mM MgCl\(_2\). It was incubated at 4°C for 30 min and then centrifuged at 40,000 x g for 30 min. The pellet obtained was finally suspended in 20 mM Mes-NaOH, pH 6.0, 15 mM NaCl and 5 mM MgCl\(_2\).

Both the normal and salt-washed PS II preparations were treated with different concentrations of trypsin (Bovine Pancreas Type III obtained from Sigma) with stirring and the reaction was stopped after 135 seconds by adding trypsin inhibitor (Soybean Type I-S, Sigma Chemical Co., USA) in a ratio of trypsin inhibitor to trypsin 2:1. Final Chl concentration was 600 µg/ml. After trypsin treatment the samples were centrifuged at 40,000 x g for 30 min and the pellet obtained was suspended in 20 mM Mes-NaOH, pH 6.0, containing 15 mM NaCl and 5 mM MgCl\(_2\) at Chl concentration of 1 mg/ml.

Oxygen evolution was measured polarographically using a Clark type electrode (Rank Brothers, UK). The assay mixture contained 20 mM Mes-NaOH, pH 6.0, 15 mM NaCl, 5 mM MgCl\(_2\), 0.4 M sucrose, 3.5 mM FeCy and 1 mM phenyl-p-benzoquinone. PS II particles equivalent to 20 µg Chl/ml were used. Illumination was provided by a 250 W tungsten halogen lamp. The sample compartment was maintained at 25°C. The reduction of FeCy was measured in Shimadzu UV 3000 spectrophotometer equipped with cross-illumination attachment. For reduction of FeCy the assay mixture consisted of 2 mM FeCy as acceptor and 6 mM NH\(_2\)OH as exogenous donor, 15 mM NaCl and 5 mM MgCl\(_2\) in 20 mM Mes-NaOH, pH 6.0. The actinic light provided by a 50 W halogen lamp was passed through a Corning CS2-62 filter and at the measuring side Corning CS4-96 filter was used. FeCy reduction was measured at 420 nm (\(E_{420} = 1 \text{ mM}^{-1} \text{ cm}^{-1}\)). PS II
particles equivalent to 10 μg Chl/ml were used.

SDS-PAGE analysis was done using Laemmli's buffer system (Laemmli, 1970). A gradient acrylamide gel (12.5 - 17.5 per cent) containing 5 M urea was used. Samples were solubilized in 62 mM Tris-HCl, pH 6.81 containing 10 per cent glycerol, 5 per cent mercaptoethanol and 40:1 (w/w) SDS/Chl, and heated for 5 min at 80°C before loading on the gel. Electrophoresis was run for 7 hours at a constant power of 4 W and the gel was stained with commasie blue R-250.

Fluorescence induction was measured in a laboratory designed instrument with $\lambda_{ex}=620$ nm and $\lambda_{det}=685$ nm. Low temperature ESR spectra were recorded in a Jeol JES-FE2XG spectrometer. The samples were placed in a 1 mm internal diameter quartz cuvette and continuous white light illumination was provided through a side opening by a 75 W Xenon lamp.

3. Results

Table 3.1 gives the relative activity of normal and salt-washed PS II. \( \text{NH}_2\text{OH} \rightarrow \text{FeCy electron transport was affected only marginally by salt-washing, whereas DCIP reduction and oxygen evolution were significantly decreased. The inhibition in the rates of oxygen evolution in salt-washed samples was restored by exogenous CaCl}_2 \) (Fig.3.1). However, in normal PS II preparations presence of exogenous Ca \( ^{2+} \) was found to be inhibitory.

The point at which calcium was added affected the activity differently. Table 3.2 shows that when Ca \( ^{2+} \) (3 mM) was added before measurement in dark, the activity was reduced by 15 per cent in normal PS II. However, when Ca \( ^{2+} \) was added during measurement (after 1 minute of illumination) a 20 per cent stimulation was seen.

Figure 3.2 shows the effect of trypsin treatment on the activity of oxygen evolution of normal and salt-washed PS II particles. On addition of trypsin (trypsin/Chl = 0.0001) the oxygen evolving activity in normal PS II preparation decreased by 25 per cent. The rate of oxygen evolution in salt-washed PS II particles did not change by trypsin treatment (Fig.3.1B) except at trypsin/Chl = 1 where a slight increase was observed.

Exogenous calcium stimulated the oxygen evolving activity in trypsin treated salt-washed preparations. In normal PS II preparations the oxygen evolving activity in presence of exogenous Ca \( ^{2+} \) was about 80 per cent of the control. However,
Table 3.1 Relative activity of salt-washed PS II particles compared to control samples.

<table>
<thead>
<tr>
<th>Activities</th>
<th>Normal</th>
<th>Salt-washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ evolution</td>
<td>100</td>
<td>28.5</td>
</tr>
<tr>
<td>H₂O ---&gt; DCIP</td>
<td>100</td>
<td>52.6</td>
</tr>
<tr>
<td>DPC ---&gt; DCIP</td>
<td>100</td>
<td>78.0</td>
</tr>
<tr>
<td>NH₂OH ---&gt; FeCy</td>
<td>100</td>
<td>95.6</td>
</tr>
</tbody>
</table>

Control activities in normal PS II preparations were: O₂ evolution, 250 μmol O₂ evolved/mg Chl/h; H₂O ---> DCIP, 288 μmol DCIP reduced/mg Chl/h; DPC ---> DCIP, 476 μmol DCIP reduced/mg Chl/h and NH₂OH ---> FeCy, 480 μmol FeCy reduced/mg Chl/h.

DCIP reduction was measured spectrophotometrically by using cross-illumination arrangement. The excitation beam was passed through Corning CS4-96 filter, and the photomultiplier was protected by using 600 nm interference filter (Carl-Zeiss, Jena). Assay mixture contained 20 mM Mes-NaOH pH 6.0, 15 mM NaCl, 5 mM MgCl₂ and 20 μg Chl/ml. The final concentrations of DCIP, DPC, NH₂OH and FeCy wherever used were 0.06 mM, 1.2 mM, 6 mM and 2 mM respectively. For O₂ evolution assay and FeCy reduction see Materials and Methods.
Fig. 3.1 Effect of exogenous CaCl$_2$ on the oxygen evolution activity in normal (●---●) and salt-washed (Δ---Δ) PS II particles. Control activity in normal PS II in absence of exogenous CaCl$_2$ was 250 μmol O$_2$ evolved/mg Chl/h.
Table 3.2 Oxygen evolution of normal PS II particles in presence of 3 mM exogenous CaCl$_2$ added before and during measurement (values given as percentage of control).

<table>
<thead>
<tr>
<th></th>
<th>O$_2$ evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Ca$^{++}$ added before measurement (in dark)</td>
<td>85</td>
</tr>
<tr>
<td>Ca$^{++}$ added during measurement (after 1 minute of illumination)</td>
<td>120</td>
</tr>
</tbody>
</table>

Control value in absence of exogenous Ca$^{++}$ was 250 $\mu$mol O$_2$ evolved/mg Chl/h.
Fig. 3.2 Oxygen evolving activity of trypsin digested normal (A) and salt-washed (B) PS II particles in absence (●----●) and in presence (○----○) of 3 mM CaCl₂. Assay mixture contained 0.4 M sucrose, 5 mM MgCl₂, 15 mM NaCl, 20 mM Mes-NaOH, pH 6.0, 1 mM phenyl-p-benzoquinone and 3.5 mM K₃Fe(CN)₆ in a final volume of 3 ml with chlorophyll concentration 20 µg/ml. Control values were 250 µmoles O₂ evolved/mg Chl/hr for normal PS II particles; 71 µmoles O₂ evolved/mg Chl/hr for salt-washed PS II particles.
addition of Ca\(^{2+}\) at trypsin/Chl = 0.0001 induced some stimulatory effect which was not present at higher concentrations of trypsin.

Figure 3.3 shows the effect of trypsin digestion on the electron transport activity from NH\(_2\)OH to FeCy in normal (Fig. 3.2A) and salt-washed (Fig. 3.2B) PS II preparations. In normal PS II preparations, very low concentration of trypsin (trypsin/Chl = 0.0001) slightly enhanced the activity while with higher concentrations of trypsin (trypsin/Chl = 0.1) there was a gradual decline in the activity. Presence of Ca\(^{2+}\) had a stimulatory effect on the rate of electron transport from NH\(_2\)OH to FeCy. In salt-washed PS II particles, lower concentrations of trypsin (trypsin/Chl = 0.0001 and 0.001) decreased the electron transport activity around 20 per cent. Stimulatory effect of Ca\(^{2+}\) was seen only at lower concentrations of trypsin (trypsin/Chl = 0.0001 and 0.001).

The polypeptide composition of trypsin digested normal and salt-washed PS II was analysed by SDS-PAGE and is compared in Fig. 3.4A and 3.4B. The intensity of protein bands at 47-43 kD, 24 kD and 16 kD decreased with increasing concentrations of trypsin. The degraded smaller protein fragments are seen with trypsin digestion at trypsin/Chl = 0.01 onwards. At the higher ratios (trypsin/Chl = 0.1 and 1) the 29-27 kD proteins of the light harvesting complex were digested resulting into polypeptides of smaller molecular weight. At trypsin/Chl = 1 the 32-34 kD polypeptides appear to be digested. In salt-washed preparation (Fig. 3.4B), the 16 kD and 24 kD proteins were absent.

The fluorescence induction (Table 3.3) of trypsin treated normal and salt-washed PS II preparation showed that with increasing trypsin concentrations the \(F_m\) value is decreased in normal and increased in salt-washed PS II.

Compared to normal PS II, the salt-washed preparation had less \(F_m\) and the rise time was also slower.

To differentiate the effect of various concentrations of trypsin on the donor and the acceptor side of PS II we studied the amplitude of the ESR signal II in dark and under continuous illumination. The results are shown in Fig. 3.5. The amplitude of both the dark stable signal and light induced signal II was reduced at trypsin/Chl = 0.0001. Higher concentration of trypsin (trypsin/Chl = 0.01) was able to restore the signal. The dark signal was restored by only 75 per cent of the control value.
The electron transport from $\text{NH}_2\text{OH}$ to $\text{K}_3\text{Fe(CN)}_6$ as a function of trypsin concentration, in normal (A) and in salt-washed (B) PS II particles in absence (•-----•) and in presence of (o-----o) of 3 mM CaCl$_2$. The 3 ml of assay mixture contained 20 mM Mes-NaOH, pH 6.0, 15 mM NaCl, 5 mM MgCl$_2$, 2 mM $\text{K}_3\text{Fe(CN)}_6$, 6 mM $\text{NH}_2\text{OH}$ and 20 μg Chl. Control activities were 480 μmoles FeCy reduced/mg Chl/hr for normal PS II particles and 458 μmoles FeCy reduced/mg Chl/hr for salt-washed PS II particles.
Fig. 3.4A Trypsin digestion of normal PS II particles as shown by SDS-PAGE in presence of 6 M urea. Lane a-standard molecular weight markers, b-thylakoid, c-normal PS II, and d-h, normal PS II treated with trypsin/Chl = 0.0001; 0.001; 0.01; 0.1 and 1. The standard Mr used were - Phosphorylase-b, 94,000; Bovine Serum Albumin, 67,000; Ovalbumin, 43,000; Carbonic Anhydrase, 30,000; Soybean Trypsin Inhibitor, 20,100 and α-lactalbumin, 14,400.
**Fig. 3.4B** Trypsin digestion of salt-washed PS II particles as shown by SDS-PAGE in presence of 6 M urea. Lane a-standard molecular weight markers b-normal PS II, c-salt-washed PS II and d-h, salt-washed PS II treated with trypsin/Chl = 0.0001; 0.001; 0.01; 0.1 and 1. The standard Mr used were - Phosphorylase-b, 94,000; Bovine Serum Albumin, 67,000; Ovalbumin, 43,000; Carbonic Anhydrase, 30,000; Soybean Trypsin Inhibitor, 20,100 and α-lactalbumin, 14,400.
Table 3.3 Relative fluorescence yield and rise time ($t_{1/2}$) in normal and salt-washed PS II particles treated with trypsin.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$F_\text{o}$</th>
<th>$F_\text{m}$</th>
<th>$F_\text{v}=F_\text{m}-F_\text{o}$</th>
<th>$t_{1/2}$ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PS II particles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>61</td>
<td>41</td>
<td>0.1</td>
</tr>
<tr>
<td>0.0001 trypsin/Chl</td>
<td>20</td>
<td>55</td>
<td>35</td>
<td>0.12</td>
</tr>
<tr>
<td>0.01 trypsin/Chl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0 trypsin/Chl</td>
<td>20</td>
<td>56</td>
<td>36</td>
<td>0.15</td>
</tr>
<tr>
<td>Salt-washed PS II particles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>37</td>
<td>17</td>
<td>0.21</td>
</tr>
<tr>
<td>0.0001 trypsin/Chl</td>
<td>20</td>
<td>39</td>
<td>19</td>
<td>0.12</td>
</tr>
<tr>
<td>0.01 trypsin/Chl</td>
<td>20</td>
<td>44</td>
<td>24</td>
<td>0.12</td>
</tr>
<tr>
<td>1.0 trypsin/Chl</td>
<td>20</td>
<td>51</td>
<td>31</td>
<td>0.15</td>
</tr>
</tbody>
</table>

The relative fluorescence intensities were measured at 685 nm with $\lambda_{\text{ex}}=620$ nm.
Fig. 3.5A  Low temperature (-150°C) ESR spectra of PS II particles treated with different concentrations of trypsin. The solid lines indicate spectra taken in dark whereas dotted lines indicates spectra taken under continuous illumination of PS II particles. The signal amplitude with respect to trypsin concentration has been plotted in Fig. 3.5B (For instrumental conditions see Fig. 3.5B).
Fig. 3.5B Effect of trypsin on the amplitude of the ESR signal II of PS II particles measured at -150°C in dark (o----o) and under continuous illumination (●--●). Magnetic field was scanned around 3360±50G. Instrumental conditions; X-band microwave region 9.15 GHz; microwave power 1 mW; field modulation intensity 1.6 G. The signal amplitude was determined at the peak obtained with lower magnetic field (3350 G).
4. Discussion

Our investigations report the effect of salt-washing and trypsin treatment on polypeptide composition, electron transport, oxygen evolving activity, fluorescence yield and ESR signal II of PS II preparation. This includes the effect of calcium.

Salt-washing results in depletion of 24 and 16 kD polypeptides (Fig.3.4B) partial inhibition of oxygen evolution and electron transport activity (Table 3.1). In presence of artificial donors NH$_2$OH and DPC, the electron transport activity of salt-washed PS II is restored. However, since the binding sites are different, there was 95 per cent restoration with NH$_2$OH as donor and only 78 per cent with DPC. Exogenous Ca$^{2+}$ is able to restore the rate of oxygen evolution (Fig.3.1).

The possible reason for more inhibition in O$_2$ evolution rates than in H$_2$O $\rightarrow$ DCIP electron transport (Table 3.1) on salt-washing, could be the difference in structural alterations in PS II membranes resulting in changes in binding affinity and accessibility of artificial acceptors (DCIP and PPBQ plus FeCy). It seems that the trypsin treatment at trypsin/Chl = 0.0001 of PS II affects the donor side. This is indicated by the decrease in the rate of oxygen evolution and its restoration by exogenous calcium (Fig.3.2). Further support comes from the electron transport activity in presence of artificial donor NH$_2$OH. The NH$_2$OH $\rightarrow$ FeCy electron transport is not influenced by trypsin and is stimulated by Ca$^{2+}$ (Fig.3.3). Higher concentration of trypsin (trypsin/Chl = 0.1 and 1) may be damaging the NH$_2$OH binding site as indicated by gradual decrease in activity. Exogenous Ca$^{2+}$ was added before measurement i.e. in dark, so that the stimulatory effect of Ca$^{2+}$ in control (without trypsin) was eliminated. Ca$^{2+}$ when added during illumination enhanced the activity of control PS II and when added in dark was slightly inhibitory (Table 3.2).

Volker et al. (1985) reported that the donor side is not affected by trypsin at pH 6.0. This could be because low concentrations of trypsin were not used by earlier workers. The SDS-PAGE analysis (Fig.34A) shows that the 16 kD protein (which is at the donor side) is digested at pH = 6.0 at low trypsin/Chl and the 32 kD protein (acceptor side) is removed only at trypsin/Chl = 1. However, partial digestion of polypeptides affecting the activity could not be assessed by the gel. Functional parameters give a combined effect of trypsin at donor and acceptor side. As seen from fluorescence induction data (Table 3.3) trypsin does not have much effect on $F_{v'}$, in normal PS II.
Our suggestion that at low concentrations of trypsin (trypsin/Chl = 0.0001) at pH 6.0, the donor side is affected, is supported by the ESR signal \( I^*_5 \) (Fig.3.5). The dark stable signal measured at -150°C was suppressed by trypsin/Chl = 0.0001. It is known that signal \( I^*_5 \) is not inhibited by the damage of acceptor side. The amplitude of the dark stable signal \( I^*_5 \) in Tris-washed or DPC-poisoned PS II is independent of FeCy concentration as well as DPC concentrations (Yuasa, et al., 1983). Only the light induced signal is affected by exogenous donors and acceptors. Therefore our results with ESR signal confirm that at trypsin/Chl = 0.0001 the donor side is affected and specifically the protein matrix of the secondary electron donor D₁ which is responsible for the signal \( I^*_5 \).

The light induced ESR signal (signal \( I^*_f \)) is attributed to the formation of \( Z^+ \), and is sensitive to inhibitory treatment of the oxygen evolving complex, particularly the extent to which Mn is associated. High salt treatment gives only 50-60 per cent of the Tris-induced light signal (Babcock, et al., 1984). The decrease in signal \( I^*_5 \) and \( I^*_f \) amplitude at low concentrations (trypsin/Chl = 0.0001) may be due to changes in protein environment at donor side and its partial restoration due to blockage of electrons at acceptor side.

Trypsin treatment in salt-washed PS II does not damage the donor side. The trypsin treatment after removal of 24 and 16 kD proteins does not affect oxygen evolution (Fig.3.2) only slightly affects \( \text{NH}_2\text{OH} \rightarrow \text{FeCy} \) electron transport (Fig.3.3) indicating a marginal damage to the donor side. As seen from the gel (Fig.3.4) trypsin digestion of 22 and 20 kD polypeptides is seen in salt-washed PS II, and not in normal PS II, suggesting that these proteins are accessible to trypsin only after salt-washing. The digestion of the 32 kD protein at trypsin/Chl = 1 corresponds to a slight increase in oxygen evolution in salt-washed PS II (Fig.3.2). This probably indicates direct accessibility of QA to artificial electron acceptors after trypsin digestion of surface exposed 32 kD QB protein which shields the QA apoprotein (Isogai, et al., 1985). The herbicide binding QB protein has also been identified as a 30 kD polypeptide (Takahashi, et al., 1986). These differences probably arise from different isolation procedures. The fluorescence data (Table 3.3) also shows increase in \( F_v \) of salt-washed PS II at this concentration of trypsin indicating blockage of electrons from QA to QB. Inhibitors on the water splitting side of P680 are known to reduce variable fluorescence and also result in slower rise time. In contrast, inhibitors on the reducing side, like in the case of DCMU result in a more rapid rise of the variable
fluorescence (Papageorgiou, 1975; Åkerlund et al., 1982). In salt-washed preparation
the increase in $F_m$ with trypsin treatment suggests that trypsin affects the
acceptor side and no further deterioration of the donor side is taking place.
In normal PS II the donor side is also affected by trypsin and the combined
effect of damage to acceptor and donor side results in practically no change
in $F_m$.

In conclusion, our observations indicate that lower concentrations of trypsin
(trypsin/Chl = 0.0001) affect the donor side and higher concentrations of trypsin
show a marked inhibition of the acceptor side of PS II particles.

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Effect of atrazine and TMPD on the photooxidation and photoreduction of different potential forms of cyt b-559 in photosystem II


Effect of ADRY reagent on photooxidation and reduction of cyt b-559 at the donor side of photosystem II

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