Identification of Flavonoids and Cinnamic Acid Derivatives from Spinach Chloroplast Preparations

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By immunochemical methods we had presented evidence for a primary acceptor complex for photosystem I of photosynthetic electron transport. During purification of this complex, obtained as a water soluble fraction from ether treated lyophilized chloroplasts, two low molecular fractions are also obtained, which may or may not have relation to the primary acceptor. The purification and identification of these low molecular fractions is reported. One, absorbing at 275 and 335 nm, consists of several flavonoid glycosides. The aglycon of the main component is tentatively identified as a 3-methyl-6,7-methylenedioxy-quercetagetin. The chromophore of the other low molecular fraction, absorbing at 312 nm, contains two cinnamic acid derivatives, which on alkaline hydrolysis yield p-coumaric acid. The relation of the two low molecular fractions to other fractions of various activity isolated from chloroplasts is discussed.

In photosynthetic electron transport of chloroplasts on illumination water is oxidized and a terminal electron acceptor is reduced. In this reaction two photosystems are involved. We have recently reported on evidence for the existence of an endogenous primary acceptor of photosystem I in chloroplasts. We had obtained an antibody against this acceptor and had isolated a fraction from ether treated chloroplasts, which would neutralize this antibody. This fraction, called $S_{L\text{-}e\text{th}}$, should therefore contain the antigen, i.e. the presumed primary acceptor. We have reported on the purification of this fraction into a high molecular weight protein, possessing the antigen activity and two low molecular fractions. This paper presents results on the isolation, separation and identification of the later compounds.

Materials and Methods

1. Isolation and Separation

The antigen fraction, called $S_{L\text{-}e\text{th}}$, was prepared from spinach chloroplasts according to REGITZ et al., but the heat inactivation step was omitted. Crude $S_{L\text{-}e\text{th}}$ (from broken chloroplasts of about 200 mg chlorophyll content) was layered on top of a Sephadex G-75 column (2.5 x 30 cm). The volumetrically collected water eluate yielded — as already described — a green fraction, a yellow high molecular fraction ($\lambda_{\text{max}}$ 275 nm), a blue fraction of plastocyanin and two low molecular yellow fractions, which came down close together. The low molecular fractions of five consecutive runs of the Sephadex G-75 column were concentrated in vacuo to about 3 ml, centrifugated and chromatographed on a Sephadex G-25 column (2 x 80 cm) with water as eluant. Fractions with absorption maxima at 260 and 310 nm (yielding low molecular fraction I — the cinnamic acid fraction) and at 275 and 335 nm (yielding low molecular fraction II — the flavonoid fraction) were collected.

Low molecular fraction I was chromatographed on a diethyl-aminoethyl cellulose column (3 x 6 cm, DE 52, Whatman). The chromophoric group with an absorption maximum at 310 nm is adsorbed, whereas a substance with an absorption maximum at 260 nm comes through the column without retardation. The column is then washed with water and then with NaCl solutions of increasing NaCl content. The chromophoric group, absorbing at 310 nm, is eluted from the column at a concentration of 0.3 M NaCl. These fractions with an absorption maximum at 310 nm are passed through a Dowex 50 W X 8 column (3 x 6 cm) for removal of Na$^+$ ions. The resulting acidic solution is lyophilized. Further purification is achieved by dissolving the residue after lyophilisation in diethyl ether, extracting the cinnamic acid derivatives into 5% NaHCO$_3$ and recovering them from the acidified water phase by repeated extraction with diethyl ether.

For the preparation of greater amounts of cinnamic acid derivatives and flavonoids the following procedure with whole spinach leaves was used. 500 g of spinach leaves were cut to pieces and boiled for 1 hour with 1 l of water. After passing the mixture through cheesecloth, the resultant filtrate was evaporated to dry in vacuo. The residue was extracted with methanol in a Soxhlet apparatus, the methanol evaporated in vacuo and the residue dissolved in 15 ml of water and acidified to pH 4. This solution was layered on top of a N-acetyl polyamide column (3.5 x 8 cm, MN Polyamide 6-AC, Macherey & Nagel) and eluted with water. A brown eluate, appearing first, is discarded. The elu-
tion of cinnamic acid derivatives is monitored at 310 nm and about 700 ml are collected. This fraction is further purified as described above.

Flavonoid compounds are then eluted with a water/methanol gradient with increasing amounts of methanol up to 100 per cent. About 2 l of yellow flavonoid solution are obtained. The eluate of flavonoids is evaporated to dry in vacuo and dissolved in a minimum amount of water. 2 ml of this solution are chromatographed on a cellulose column (3 × 90 cm, Avicel Merck) with 15% acetic acid. Elution is monitored at 335 nm. Four fractions are obtained. Corresponding fractions of several consecutive runs are combined and lyophilized.

2. Hydrolysis and Chemical Procedures

Acid hydrolysis of flavonoids was carried out by boiling 2 hours with 6% HCl and extracting the aglycon with ethyl acetate.

Cleavage of aromatic ether linkages was achieved by boiling 1 hour with equal amounts of freshly distilled 67% hydroiodic acid and acetic anhydride. After evaporation of the acetic anhydride in vacuo and dilution with water the flavone was extracted into ethyl acetate. Elemental iodine in the ethyl acetate phase was reduced by shaking with a dilute solution of sodium thiosulphate and thus removed from the ethyl acetate phase.

Quercetagetin was obtained from the hexamethyl ether * in the same way.

Alkaline hydrolysis of cinnamic acid derivatives was brought about by stirring with 2 N NaOH at room temperature for 4 hours under nitrogen atmosphere. After acidification to pH 3, the cinnamic acid was extracted with diethyl ether, re-extracted into 5% NaHCO₃ and recovered from the NaHCO₃ phase by repeated extraction with diethyl ether prior to acidification.

p-coumaric acid methyl ester was synthesized according to Harborne and Corner 4.

Catalytic hydrogenation was achieved with palladium-charcoal as catalyst.

3. Thin Layer Chromatography

The following solvent systems were employed (all mixtures by volume, if not indicated otherwise):

Cellulose (DC-Fertigplatten, Merck): a: tert. butanol-acetic acid-water 3:1:1; b: 15% acetic acid; c: n-butanol-acetic acid-water 4:1:5 (upper phase); d: water; e: n-butanol-2 N NH₃ 1:1 (upper phase); f: n-butanol-ethanol-water 4:1:2.2; g: benzene-acetic acid-water 6:7:3 (upper phase); h: sodium formate-formic acid-water (10 g : 1 ml : 200 ml); j: 2% acetic acid.

Polyamide (Maderrey & Nagel, Polygram Fertigfolien Polyamid-6) k: methanol-acetic acid-water 90:5:5.

Chromatograms were examined in UV-light of 350 nm, in the presence or absence of ammonia vapors.

Flavonoids were sprayed with Benedikt's reagent and cinnamic acids with 2% diazotized sulfanilic acid in 10% Na₂CO₃.

4. Spectroscopy

UV-spectra were determined in a Cary 15 spectrophotometer. The procedure of MABRY et al. 5 was used to obtain diagnostic shifts of flavonoid spectra on addition of various reagents. Mass spectra were determined in a Atlas CH 7 mass spectrometer. Trimethylsilylation was achieved by adding a few μl of bis-(trimethylsilyl)acetamide to the sample, prepared for mass spectrometry, and heating to 100° for a few minutes.

Results

As has been reported already, the crude antigen fraction S₅-eth, which neutralizes the antibody inhibition of the presumed primary acceptor of photosystem I, can be separated on a Sephadex G-75 column into several fractions 3. The two low molecular fractions thus obtained may or may not be related to this primary acceptor. Their further purification is described in methods. As will be documented in the following tables and figures, we have identified the compounds in low molecular fraction I as cinnamic acid derivatives and in the low molecular fraction II as a mixture of flavonoid compounds.

1. Low molecular fraction II — flavonoids

Two dimensional thin layer chromatography of the purified low molecular fraction II revealed the presence of nine flavonoid compounds. Rₗ-values are given in Table 1. Compounds, only present in traces, are set into paranthesis.

<table>
<thead>
<tr>
<th>Designation of the compound</th>
<th>Rₗ-values in solvent systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>A</td>
<td>0.29</td>
</tr>
<tr>
<td>(B1)</td>
<td>0.29</td>
</tr>
<tr>
<td>B2</td>
<td>0.39</td>
</tr>
<tr>
<td>C1</td>
<td>0.24</td>
</tr>
<tr>
<td>C2</td>
<td>0.40</td>
</tr>
<tr>
<td>(D1)</td>
<td>0.24</td>
</tr>
<tr>
<td>(D2)</td>
<td>0.34</td>
</tr>
<tr>
<td>(D3)</td>
<td>0.37</td>
</tr>
<tr>
<td>(D4)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Table 1. Rₗ-values of flavonoids from low molecular fraction II in thin layer chromatography (solvent systems are described in methods).

The final column chromatography on the cellulose column yields four fractions. They correspond
to the following compounds in thin layer chromatography (in the order of elution): D 1—D 4, C 1 and C 2, B 1 and B 2 and finally A. Compound A proved to be chromatographically pure and identification was started with this compound. As rational for the identification procedure it was assumed that compound A ($\lambda_{\text{max}}$ (methanol) 250, 276 and 338 nm) is a glycosidated flavonoid. Acid hydrolysis yielded 1, whose absorption spectrum could not be referred to a common flavone. Additional substituents, especially ether linkages, were assumed. Therefore further cleavage with hydroiodic acid was carried out, yielding the basic flavonol 2. Mass spectrometric analysis, done by Dr. D. MÜLLER (Abt. Chemie, Ruhr-Universität Bochum), of the trimethylsilyl derivative of 2, showed it to be a hexahydroxy flavone. By comparison with an authentic sample, the structure of quercetagetin could be established for 2 ($R_f$-values in solvent systems a: 0.17; b: 0.02 and k: 0.17). $\lambda_{\text{max}}$ (methanol) 257, 273 and 356 nm are in good agreement with the data $\lambda_{\text{max}}$ (ethanol) 259, 272 and 364 nm, reported by HARBORNE.

The presence of a methyl and a methylenedioxy group was established in 1 by mass spectrometric analysis, again done by Dr. D. MÜLLER. The mass spectrum of the tris(trimethylsilyl) derivative of 1 is shown in Fig. 1; only peaks >350 m/e are indicated.

Neglecting an impurity at 563 m/e, 560 m/e proved to be the molecular ion. As known from the mass spectroscopy of trimethylsilyl compounds, a methyl group is split off very easily, yielding the fragment ion 545 m/e. The high intensity of 545 m/e, compared to the molecular ion, is due to the formation of a six membered silyl ring and a very stable aromatic system.

488 m/e, which is the base peak in this spectrum, is not due to an additional fragment ion, but to the bis(trimethylsilyl) derivative of 1 with one free hydroxyl group. As is known from methylation, the strongly hydrogen bonded hydroxyl group in the 6 position is only substituted with difficulties. The corresponding fragment ion after loss of a methyl group, 473 m/e, has only very low intensity, because a stabilizing effect by ring closure is impossible in this case. Additional proof for the presence of a methyl and a methylenedioxy group in 1 was given by high resolution mass spectrometry. The molecular weight found was within the value of 0.009 mass units for the exact calculated molecular weight of 1. Further information, concerning the position of the above substituents, could not be obtained from this mass spectrum. No additional characteristic fragment ions could be observed.

The substitution pattern is tentatively established by UV-spectroscopy ($\lambda_{\text{max}}$ of 1 in methanol: 258, 268, 275 sh and 350 nm). A shift of 17 nm of band I, produced by boric acid/sodium acetate, postulates the presence of two free ortho hydroxyl groups in the 3' and 4' position. A shift of 22 nm of band I, caused by aluminium chloride/hydrochloric acid, indicates the presence of a free hydroxyl group in the 3 or 5 position. Since 1 is stable on addition of sodium methylate, a free hydroxyl group in the 3 position is excluded. Thus, the structure of 1 is tentatively established as 3-methyl-6,7-methylenedioxy-quercetagetin.

Nature and position of the substituent, which is split off on acid treatment from A, i.e. the original compound in the low molecular fraction II, are not yet known.

Information on the remaining compounds B 1 and B 2 and C 1 and C 2 (the compounds of the D series were omitted, because these components were only present in traces), which are not yet resolved into single components, could be obtained by chromatographic comparison of their hydrolysis products with those of A, as shown in Table 2.
Table 2. Comparison of the hydrolysis products of compounds from low molecular fraction II in thin layer chromatography (solvent systems are described in methods).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>hydrolysis 6% HCl</th>
<th>cleavage hydroiodic acid</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R_f-values in solvent systems</td>
<td>R_f-values in solvent systems</td>
</tr>
<tr>
<td>A</td>
<td>a 0.65, b 0.04, k 0.39</td>
<td>a 0.16, b 0.02, k 0.17</td>
</tr>
<tr>
<td>B1/B2</td>
<td>a 0.66, b 0.04, k 0.39</td>
<td>a 0.18, b 0.02, k 0.17</td>
</tr>
<tr>
<td>C1/C2</td>
<td>a 0.78, b 0.11, k 0.46</td>
<td>a 0.18, b 0.02, k 0.17</td>
</tr>
</tbody>
</table>

Table 3. Comparison of R_f-values of the alkaline hydrolysate of the chromophoric group of low molecular fraction I with that of p-coumaric acid in thin layer chromatography with authentic p-coumaric acid.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>p-coumaric acid</th>
<th>hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>0.37</td>
<td>0.40</td>
</tr>
<tr>
<td>h</td>
<td>0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>i</td>
<td>0.89</td>
<td>0.90</td>
</tr>
<tr>
<td>j</td>
<td>0.37</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Identity of p-coumaric acid in the alkaline hydrolysate of the chromophoric group of low molecular fraction I is confirmed by its catalytic hydrogenation to phloretic acid and comparison with authentic phloretic acid, obtained from p-coumaric acid in the same way (R_f-values in solvent systems g: 0.41 and h: 0.73; purple violet colour reaction with diazotized sulfanilic acid, λ_max 277, 285 sh nm).

The substituent, which is split off by alkaline hydrolysis from both compounds, must be linked to the carboxylic group of p-coumaric acid. The hydroxyl group must be free, otherwise the bathochromic shift of 46 nm on addition of alkali cannot be explained. The methyl ester of p-coumaric acid...
shows quite the same UV-spectrum as the mixture of both cinnamic acid derivatives isolated, which supports the assumption that the carboxylic group of p-coumaric acid is esterified by a compound without a marked selfabsorption in both cases. The chemical nature of these substituents is not yet known.

Discussion

The terminal electron acceptor of the photosynthetic electron transport chain in chloroplasts is the water soluble ferredoxin, which connects the reducing power of photosystem I to NADP\(^{+}\)-reduction. Since there are numerous Hill reactions independent of the addition of ferredoxin, and since viologen dyes with much more negative redox potentials than ferredoxin are reduced by isolated chloroplasts, it was speculated that there is a primary acceptor of photosystem I different from ferredoxin. Recently evidence has been presented for the existence of such a compound between photosystem I and ferredoxin. YOCUM and SAN PIETRO\(^{6,9}\) discovered a substance, called FRS — ferredoxin reducing substance — which in addition to ferredoxin is required to restore NADP\(^{+}\)-reduction in highly sonicated chloroplasts.

We have presented evidence that an antiserum against the thylakoid system of chloroplasts contains an antibody against the unknown primary acceptor of photosystem I\(^{1,2}\). REGITZ\(^{2,3}\) obtained an extract from ether treated lyophilized chloroplasts, called \(S_{L-\text{eth}}\), which would neutralize the inhibitory effect of the antibody preparation and therefore should contain the antigen, i.e. the presumed primary acceptor.

REGITZ\(^{3}\) has recently reported on the purification of the protein fraction from \(S_{L-\text{eth}}\), which carries the antigen property. In the crude \(S_{L-\text{eth}}\) also two low molecular fractions are present. The results in this paper have identified one as a mixture of several flavonoid glycosides and the other as two derivatives of p-coumaric acid. The main compound in the flavonoid fraction is tentatively characterized as a 3-methyl-6,7-methylenedioxy-quercetagetin glycoside. The two derivatives of p-coumaric acid are esterified with not yet identified compounds. Quinic acid or glucose, which are the most common compounds esterified with p-coumaric acid in nature, do not seem to be involved. It seems possible that our derivatives of p-coumaric acid isolated from chloroplasts, are related to or identical with the recently described p-coumaryl-meso-tartaric acid and its acetyl derivative, isolated from spinach leaves\(^{9a}\).

At present, it is not possible to relate the two low molecular fractions to the antigen moiety in our \(S_{L-\text{eth}}\) preparation. Since the protein purified by REGITZ\(^{3}\) from \(S_{L-\text{eth}}\) already neutralizes the antibody against the primary acceptor, we have at present no means to show that one of the low molecular fractions had been dissociated from the protein fraction during purification and therefore is perhaps the prosthetic group of the primary acceptor. As has been pointed out by REGITZ et al.\(^{2}\), the antibody not necessarily must be directed against the prosthetic group of the primary acceptor and therefore the antigen may be the apo-enzyme or even only a structural component of the primary acceptor complex. As already discussed\(^{2,3}\), the designation of the antigen isolated from \(S_{L-\text{eth}}\) as the primary acceptor of photosystem I and an electron carrier rests on its relation or part identity to FRS of YOCUM and SAN PIETRO.

It is interesting to note, however, that SAN PIETRO\(^{10}\) in a recent report on purification of FRS also obtained low molecular fractions, which he has not yet identified, but which as judged from their absorption spectra, seem to be identical with our low molecular fractions. It seems to be more than a coincidence that the low molecular fractions are obtained from chloroplasts in completely different preparations, but both containing the presumed primary acceptor.

The two low molecular fractions, we have obtained from \(S_{L-\text{eth}}\), show similarities to other compounds reported in the literature.

CRS — cytochrome c reducing substance — isolated by FUJITA and MYERS\(^{11}\) and FUJITA and MURANO\(^{12}\) shows great resemblance to FRS of SAN PIETRO and to our \(S_{L-\text{eth}}\). It has been suggested by the authors that CRS might be related to the primary acceptor of photosystem I\(^{11,12}\). REGITZ et al.\(^{2}\) reported that \(S_{L-\text{eth}}\) has CRS activity.

A number of naturally occurring cofactors of photosynthetic phosphorylation have been isolated from chloroplasts. The cofactors, isolated by KROGMANN and STILLER\(^{13}\) and GEE et al.\(^{14}\), are reported to be of flavone type of unknown structure. They correspond to the quercetagetin derivatives of low molecular fraction II from \(S_{L-\text{eth}}\). “Phosphodoxin”,
a preparation by BLACK et al.\textsuperscript{15}, exhibits similar absorption maxima in the regions of 260 – 280 and 315 – 330 nm, as do both low molecular fractions from \( S_{L-Eth} \). The active compound of “phospho-
doxin” is claimed to be a pteridin\textsuperscript{16, 17}. No pteri-
din, however, could be detected in low molecular fractions of \( S_{L-Eth} \). A fluorescent protein factor, iso-
lated by WU and MYERS\textsuperscript{18} and its phosphorus con-
taining chromophoric group (“P-compound”)\textsuperscript{19} both stimulate photophosphorylation. Identification of “P-compound” has not yet been achieved, but a number of characteristics of unconjugated pteri-
dines is reported\textsuperscript{19}. UV-data, fluorescence behaviour and chemical properties of “P-compound”, as re-
ported by Wu et al.\textsuperscript{19}, are quite similar to those of the p-coumaric acid derivatives, we obtained from low molecular fraction I from \( S_{L-Eth} \). We think it possible therefore that both are identical and therefore “P-compound” should be considered as a p-coumaric acid derivative.

There also might be some connection between the primary acceptor of photosystem I and the light activation factor of WILDNER et al.\textsuperscript{20}. Ribulose-
diphosphate carboxylase from tomato plants shows a stimulation of activity upon irradiation with light of 325 nm. Responsible for light activation is a protein with a chromophore, absorbing at 325 nm and identified as chlorogenic acid\textsuperscript{20}. But the possibility cannot be ruled out that another compound with similar spectroscopic properties (i.e. a p-coumaric acid derivative) but in low concentrations is masked by chlorogenic acid, which is one of the most common phenolics in plants\textsuperscript{20}.

As stressed already, we have not yet shown that the two low molecular fractions in \( S_{L-Eth} \) are related to the protein moiety with the antigen activity and therefore to the primary acceptor. However, we can rule out pteridines being related to the primary ac-
ceptor, because we find no evidence for a pteridine in our preparation \( S_{L-Eth} \). Strong suggestions that a pteridin might act as the prosthetic group of the primary acceptor of photosystem I had been made by FULLER and NUGENT\textsuperscript{21}, based on the observations that the presence of pteridines in spinach had been established\textsuperscript{16} and that the redox potentials of tetrahydropterines as measured by polarographic methods seemed to be negative enough to reduce ferredoxin\textsuperscript{21}. However, recently it has been shown that the hitherto reported redox potentials for the quinonoid dihydropterin-tetrahydropterin couple of \(-0.7\ \text{V}\) should be revised. ARCHER and SCRI-
MEDGEUR\textsuperscript{22} reexamined the standard reduction poten-
tial and reported a \( E_0^{\circ} \)-value of \(+0.15\ \text{V}\). REED and

MAYNE\textsuperscript{23} could not find any pterin in the fully active reaction centers of Rhodopseudomonas spheroides.

Recently MALKIN and BEARDEN\textsuperscript{24} suggested that a bound ferredoxin may be the primary acceptor of photosystem I and HIYAMA and KE\textsuperscript{25} reported on a spectral change at 430 nm attributed to the primary acceptor of photosystem I. At present it is not possible to correlate their data with ours and those of SAN PIETRO.

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gemeinschaft”. We are indebted to Prof. Dr. A. TREBST for many helpful discussions and to Dr. D. MÜLLER for mass spectrometric analysis.

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Über Teilreaktionen der Photosynthetischen Sulfatreduktion in zellfreien Systemen aus Spinatchloroplasten und Chlorella

Enzyme Reactions Involved in Photosynthetic Sulfate-Reduction in Cell-free Systems of Spinach Chloroplasts and Chlorella

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Sulfatreduktion, APS, PAPS, S-Sulfoglutathion

Cellfree extracts from Chlorella, spinach leaves or spinach chloroplasts convert radioactive sulfate into a S-sulfocompound already in the dark. The reaction requires ATP, Mg$^{2+}$ ions and compounds with SH groups. The later are needed for activation of the enzyme and as substrates and acceptors for the sulfogroup. The compound formed if glutathion is used has been identified as S-sulfoglutathion. Other products formed from sulfate and ATP in the dark by spinach or chlorella extracts are APS and PAPS. The formation of PAPS requires the presence of a SH-group. Both cellfree extracts transfer the sulfate group from APS and PAPS onto glutathion to form S-sulfoglutathion. No ATP is needed in this reaction, neither with PAPS nor with APS as substrate.

Methodik

a) Chloroplastenextrakt: Spinatchloroplasten, nach der Methodik von JENSEN und BASSHAM$^{10}$ hergestellt, wurden bsmotisch aufgebrochen und der würzige Überstand (entsprechend einer Chlorophyllkonzentration von 0,6 mg/ml) als Enzympräparation benutzt$^{4}$. 

b) Chlorella-Extrakt: Chlorella pyrenoidosa wurde nach der Vorschrift von BÖGER$^{11}$ ohne Vitaminzusatz angezogen und nach einem abgeänderten Verfahren von SCHIFF in der French-Presse aufgebrochen. Die Kulturlösung werden abzentrifugiert und in 0,02 M Tris-HCl-Puffer pH 7,8 (pro 1 werden 200 mg GSH zugegeben) so aufgenommen, daß zu 1 g Chlorella-Zellen (Naßgewicht) 5 ml Puffer gegeben werden. Diese Lösung wird in der French-Presse (Sorvall Ribi RF I) bei 20000 PSI aufgebrochen, wobei die Temperatur zwischen 10 und 15 °C gehalten wird. Dieser so gewonnene Rohextrakt wird sofort in Portionen zu je 10 ml eingefroren. Zum Versuch wird jeweils eine Portion aufgetaut und für 10 Min. bei 10000 g abzentrifugiert. Durch eine Sephadex-G-50-Säule (1,7-25 cm) werden alle niedermolekularen Verbindungen abgetrennt (0,02 M Tris-HCl-Puffer pH 7,8 mit