Purification and kinetic properties of the urease inhibitor complex from poplar leaves (*Populus berolinensis*)

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1. Purified preparations of the apo-inhibitor and co-inhibitor have been prepared. 2. The inhibition is of the noncompetitive type and is irreversible. 3. L(+)-cysteine, reduced glutathione and 2-mercaptoethanol prevent completely the inhibition, when added in excess to the preincubation mixture, but are unable to reverse inhibition, once the enzyme-inhibitor complex has been formed. 4. The inhibitor complex catalyses the oxidation of reduced glutathione and after a lag of four hours, inhibition can be fully restored. 5. The mechanism of the inhibition has been discussed.

We have previously reported about the presence of an urease inhibitor in poplar leaves (1,2). The inhibitor complex appeared to be composed of 2 substances, neither of which caused inhibition by itself. In this paper, methods of partial purification of the 2 components of the inhibitor complex are described and some data concerning its kinetic properties in relation to urease activity reported.

MATERIAL AND METHODS

Fresh poplar leaves were air dried for 3—4 days, finely ground and stored in a cool room at -5° . The powder lost none of its activity over a period of 12 months.

Preparation of the co-inhibitor: 10 g of the ground leaves were immersed in 200 ml of boiling redistilled water. After heating at 100° for 15 minutes, the liquid was cooled and filtered through a plug of cotton wool. The residue was washed with water to a total volume of 200 ml. The extract was then evaporated on a boiling water bath to about 20 ml and dialysed against 50 ml of redistilled water in the cool room, protected from direct sunlight for about 20 hours. The dialysate was concentrated on a boiling water bath to 20 ml and on cooling 2 g of DEAE-Sephadex A-50 medium were stirred into the solution. 20 ml of redistilled water were added to the thick brei and the solution was filtered through a hard filter paper. The residue was washed twice with 20 ml of water and the combined solutions again evaporated as before to a volume of 1 — 2 ml. This concentrated extract was placed on top of a column filled with Sephadex G-25 fine (35×1.8 cm) and eluted

with redistilled water at a speed of 7—8 seconds per drop. The first 75 ml were discarded. The following 25 ml of the eluent were collected and again evaporated as before to 1—2 ml. Gel filtration on a Sephadex bed as described above was repeated, except that the first 80 ml were discarded and the following 20 ml of the eluate collected. The eluate was then evaporated under reduced pressure at room temperature over NaOH and a resinous yellow coloured product was obtained. From 10 g of dried leaves about 15 mg of the product were generally obtained.

Preparation of the apo-inhibitor: 20 g of the ground leaves were well macerated in a mortar with 100 ml of a 1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution in redistilled water. To this mixture further 300 ml of the 1% magnesium sulphate solution were added and the lot was incubated under occasional stirring for 1 hour at 45°. On rapid cooling the solution was centrifuged in the cool room at 12.000 rpm (Servall centrifuge, powerstat setting 220). The supernatant was decanted, filtered through cotton wool and immediately precipitated with solid $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Per each 100 ml of the supernatant, 45 g of the finely powderised salt was added in small portions. When the salt was entirely dissolved the resulting protein precipitate was centrifuged off for 15 minutes at 12.000 rpm. The supernatant was discarded and the tubes drained for 10 minutes. 5 ml of ice cold water was added to one of the centrifuge tubes, the content well stirred with a glass rod and the liquid plus undissolved precipitate transferred into the next tube. When the content of all the tubes were thus treated and washed into the last tube, washing was repeated again with 5 ml of redistilled water as above. The combined washings were centrifuged for 10 minutes at 12.000 rpm, the supernatant carefully decanted and each 5 ml placed on a bed of Sephadex G-25 fine (35×1.8 cm). Redistilled water was used as eluent. On gel filtration at a speed of 7—8 seconds per drop, a broad greyish band was formed at the head of the column. 8 ml of this eluate were collected and the solution evaporated under reduced pressure in a dessicator over NaOH. A dry brownish amorphous product was obtained with a yield of about 25 mg per 20 g of dried leaves. The whole procedure was carried out in the cool room at +5°. Owing to sharply rising instabilities of the co-inhibitor and apoinhibitor, further purification has been temporarily halted.

Determination of urease inhibition

Reagents:

1. Urease. 1 mg of urease (3.5.1.5) lyophil. reinst, Serva Entwicklungslabor, Heidelberg, Germany, was dissolved in 5 ml of 0.5% gelatine solution. Owing to slow oxidation by air, the solution should be prepared freshly each day and kept in the cool room.

2. Urea. 1% solution in 0.04 M potassium phosphate buffer pH 6.9. Must be prepared freshly each day.

3. Apo-inhibitor. 1 mg of the active protein preparation was dissolved in 2 ml of 0.5% gelatine solution. If stored in the cool room, this solution may be kept for several days. When smaller concentrations are required, the corresponding dilutions must be prepared just before use.

4. Co-inhibitor. 6 mg of the preparation are dissolved in 100 ml of redistilled water. This solution protected from direct sunlight and stored at +5° was stable for several weeks. More dilute solutions must be prepared freshly before use.

5. Buffer solution. 0.2 M potassium phosphate pH 8.0.

6. Hydrochloric acid. About 1 N HCl.

7. Nessler reagent. 37.5 g KI and 50 g HgI₂ were dissolved in 25 ml of water and made up to 500 ml. To this, 2330 ml of a 10% solution of NaOH were added, followed by another 500 ml of water. After standing for 24 hours, the solution is ready for use.

Determination: 50 μ l of redistilled water, 5 μ l of the buffer, 50 μ l of urease solution, followed by 1 μ l of the apo-inhibitor and 1 μ l of the co-inhibitor were pipetted into small test tubes (10 $\times$ 1 cm), preferably in the cool room, stoppered and preincubated at 30° for 1 hour. After preincubation, 1 ml of the urea solution was quickly added, the tubes stoppered and the mixture incubated again for 1 hour at 30°. Then 4 ml of the hydrochloric acid were rapidly added, the content washed quantitatively into 50 ml measuring flasks and filled to the mark with distilled water. Aliquots of 4 ml of this solution were pipetted into test tubes containing 1 ml of the Nessler reagent and the ammonia determined colorimetrically at 420 m μ . Blanks were determined in the same way, except that the 4 ml of HCl were added prior to incubation with urea. Total urease activity was estimated as above, but no inhibitor complex was added. Ammonia-N was read from a curve prepared on nesslerisation of 5—100 μ g of (NH₄)₂SO₄.

RESULTS AND DISCUSSION

For the determination of the inhibition curves extremely small quantities of the apo-inhibitor as well as the co-inhibitor were required. The necessary dilutions could not be prepared, as the apo-inhibitor and the co-inhibitor were then very unstable and erratic results were obtained. For this reason it was essential to employ much stronger urease solutions and use much smaller aliquots of the incubate for the determination of ammonia. This was done in the following manner: 4 mg of urease were dissolved in 1 ml of 0.5% gelatine and a 5% urea solution was prepared in 0.04 M potassium phosphate buffer, pH 6.9.

For the apo-inhibitor titration curve, 1 mg of the apo-inhibitor was dissolved in 4 ml of 0.5% gelatine and 20, 10, 8, 6, 4, 2, 1 μ l respectively were used. In all cases excess of the co-inhibitor was added (20 μ l). For the co-inhibitor titration curve 7, 6, 5, 4, 3, 2, and 1 μ l respectively of the co-inhibitor solution were used in presence of excess of apo-inhibitor

Fig. 1. Inhibition as function of apo-inhibitor concentration at excess level of co-inhibitor (0.005 μ g).

Ammonia-N produced in 1 hour at 30°: 6.3 μ g.

Fig. 2. Inhibition as function of co-inhibitor concentration at excess level of apo-inhibitor (0.02 μ g).

Ammonia-N produced in 1 hour at 30°: 7.7 μ g

(20 μ l). All further procedure was exactly as described before, except that after dilution of the incubate to 50 ml, only 0.2 ml made up with water to 4.0 ml were taken for nesslerisation. As can be seen from fig. 1 and 2, as little as 0.318 μ g of apo-inhibitor or 0.0218 μ g of the co-inhibitor were necessary to obtain 100% inhibition per 100 μ g of ammonia-N produced.

Assuming tentatively that the molecular weight of the apo-inhibitor was 10,000, then 3.18×10^{-11} mols caused full inhibition. If the molecular weight of the co-inhibitor was to be 44 (see below), then the corresponding figure for the co-inhibitor was 5×10^{-10} mol. Preincubation of urease with the inhibitor complex was essential in order to obtain maximum inhibition and fig. 3 shows that 1 hour was optimal time and that the effect of temperature was nearly negligible.

Fig. 3

Fig. 4

Fig. 3. Time of preincubation at excess level of apo-inhibitor (0.5 μ g) and co-inhibitor (1.0 μ g).

preincubation at 30° — — —; preincubation at 5° ———

Ammonia-N produced in 1 hour at 30°: 120 μ g.

Fig. 4. Effect of pH on inhibition during preincubation period. Excess of apo-inhibitor (0.5 μ g) and co-inhibitor (1.0).

Ammonia-N produced in 1 hour at 30°: 144 μ g.

For the formation of the enzyme-inhibitor complex during the preincubation period pH was of considerable importance. 100% inhibition was obtained at pH 8.0, but below neutrality this effect declined rapidly, (fig. 4). In all subsequent work therefore, the time of preincubation was maintained at 1 hour at 30° and the pH was adjusted to 8.0. L(+)-cysteine, reduced glutathione (GSH) and 2-mercaptoethanol prevented entirely the inhibition of urease (fig. 5, 6, 7, 8).

Fig. 5

Fig. 5. Effect of cysteine on inhibition, added before and after preincubation.
 Excess of apo-inhibitor ($0.5 \mu\text{g}$) and co-inhibitor ($1.0 \mu\text{g}$).
 Ammonia-N produced in 1 hour at 30° : $100 \mu\text{g}$

Fig. 6

Fig. 6. Effect of cysteine titration on deinhibition in presence of excess apo-inhibitor ($0.5 \mu\text{g}$) and co-inhibitor ($1.0 \mu\text{g}$).
 Ammonia-N produced in 1 hour at 30° : $154 \mu\text{g}$

Fig. 7

Fig. 7. Effect of GSH titration on deinhibition in presence of excess apo-inhibitor ($0.5 \mu\text{g}$) and co-inhibitor ($1.0 \mu\text{g}$).
 Ammonia-N produced in 1 hour at 30° : $125 \mu\text{g}$
 Extrapolation if GSH would not be oxidised — — —

Assuming that 1 mol of compound with the SH group reversed quantitatively the inhibition of 1 mol or its multiple of the co-inhibitor which is reasonable, as the full deinhibition was obtained with equimolecular concentrations of 2-mercaptoethanol as well as GSH, then the minimum molecular weight of the co-inhibitor would be 44:

Fig. 8

Fig. 9

Fig. 8. Effect of 2-mercaptoethanol titration on deinhibition in presence of excess apo-inhibitor (0.5 μg) and co-inhibitor (1.0 μg).
Ammonia-N produced in 1 hour at 30°: 127 μg

Fig. 9. Plot of $1/V$ vs $1/S$ according to Lineweaver-Burk. For apo-inhibitor plot 0.2 $\mu\text{g/ml}$ apo-inhibitor at excess of co-inhibitor was used. Correspondingly for the co-inhibitor plot 0.012 $\mu\text{g/ml}$ co-inhibitor was used at excess of apo-inhibitor.

$$\text{m.w. of co-I} = \frac{\text{m.w. of GSH} \times \mu\text{g co-I}}{\mu\text{g GSH}} = \frac{307,33 \times 0,0218}{0,015} = 44$$

As can be seen from the plot according to Lineweaver and Burk, the inhibition is noncompetitive (fig. 9).

Read from fig. 9: — $K_m = 0.06$, therefore $K_m = 1.67 \times 10^{-2}$ Mol.

K_I calculated from the equation:

$$K_I = \frac{(I)y}{x-y} = \frac{0,0002 \times 0,08}{0,11 - 0,08} = 5,3 \times 10^{-4} \text{ g/l}$$

where (I) = apo-inhibitor concentration; y = point of intersection of the "no inhibitor" plot with the ordinate; x = point of intersection of the apo-inhibitor plot with the ordinate. Assuming the m. w. of the

apo-inhibitor was 10.000, then $K_{apo-I} = 5.3 \times 10^{-8}$ Mol. Correspondingly assuming the m. w. of the co-inhibitor to be 44, then

$$K_{co-I} = \frac{0,000012 \times 0,08}{0,17 - 0,08} = 1,07 \times 10^{-6} \text{ g/l or } 2,43 \times 10^{-7} \text{ Mol}$$

There was no relationship between the degree of inhibition and concentration of the substrate. Inhibition depended only on the concentration of the inhibitor complex.

Fig. 10. Effect of time on oxidation of GSH in presence and in absence of the inhibitor complex during preincubation, expressed in % of reestablished inhibition. Excess of apo-inhibitor (0.5 μ g) and co-inhibitor (1.0 μ g)
Ammonia-N produced in 1 hour at 30°: 145 μ g

Noncompetitive inhibition may be due to combination of the inhibitor with the same site of the enzyme at which the substrate would combine, or of the combination of the inhibitor with some portion of the enzyme molecule not directly involved in the enzyme-substrate complex, but which may lead to conformational changes, so that catalytic activity is hindered. As may be seen from fig. 6, 7 and 8, cysteine, 2-mercaptoethanol and GSH prevented inhibition, when added to the enzyme before the inhibitor-enzyme complex was formed. On the other hand, when the inhibitor-enzyme was already established, the inhibition proved to be irreversible even on addition of large excess of thiol compounds (fig. 5).

Fig. 10 shows that the action of GSH could be reversed in time, but only due to oxidation of the SH group to S-S.

The reappearance of inhibition in time despite the presence of GSH in the preincubation mixture must have been due to oxidation of the SH groups, because addition of cystine or GSSG had no effect on inhibition. This is also proved indirectly by the action of cysteine, of which much larger quantities had to be used to obtain full deinhibition, owing to the fact, that cysteine is quickly oxidised in the alkaline medium in which preincubation was generally conducted.

The inhibitor complex catalised the oxidation of GSH, as after 4 hours of standing of the mixture at 5°, followed by addition of urease and urea, 100% inhibition has been reestablished. On the other hand no oxidation of GSH at pH 8.0 by itself has been observed, because on addition after 4 hours of the inhibitor complex, urease and urea, full urease activity was detected.

It would therefore appear that the conformational change of the urease molecule due to the presence of SH groups, can be reversed to the original conformation of the molecule, concurrent with the oxidation of the GSH to GSSG. Thus the enzyme-inhibitor complex can be formed again and prevent the hydrolysis of urea. This may suggest that the SH group competes for the same active centre of the enzyme as does the inhibitor and that the SH group has greater affinity for the active site of urease. However when the enzyme-inhibitor complex has once been established, then the SH group cannot break it up, urea cannot enter the molecule of urease. It may be that there are two active centres in close proximity, one for the SH group and one for urea, both being blocked by the inhibitor complex.

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*Oczyszczenie i niektóre kinetyczne własności inhibitora ureazy z liści topoli
(Populus berolinensis)*

Streszczenie

1. Otrzymano częściowo oczyszczone preparaty apo-inhibitora i ko-inhibitora ureazy.
2. Inhibicja jest typu niewspółzawodniczego i jest nieodwracalna.
3. W obecności L(+)-cysteiny, zredukowanego glutationu i 2-merkaptoetanolu kompleks enzym-inhibitor nie zostaje utworzony i hydrolityczny rozkład mocznika przebiega normalnie.
4. Utworzony kompleks enzym-inhibitor nie ulega rozpadowi w obecności związków zawierających grupę SH, jeżeli te zostały dodane po preinkubacji.
5. Kompleks inhibitora katalizuje utlenianie zredukowanego glutationu, który w postaci utlenionej, podobnie jak cystyna nie przeciwdziała inhibicji.
6. Przedyskutowano przypuszczalny mechanizm inhibicji ureazy.