A New Method for the Preparation of Jack-bean Urease Involving Covalent Chromatography

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Urease (EC 3.5.1.5) can be prepared from jack-bean meal by a simple extraction crystallization procedure devised by Sumner.[1] The method, which does not reproducibly give urease preparations of high specific activity and homogeneity, has been improved by a number of additional separation steps.[2,3] The extended procedures, however, tend to be rather laborious and time consuming.

Recently we have developed a method for the immobilization of urease by thiol–disulfide exchange.[4] An agarose-2-pyridyl-disulfide gel was used as activated solid support. Since the immobilization of partially purified urease was followed by a 6–10-fold purification and since the immobilization is reversible, it was proposed that the technique could be used for preparation of urease. The same technique, known as covalent chromatography by thiol-disulfide exchange, has recently been used to prepare papain from papaya latex and mercaptalbumin from bovine serum albumin.[5,6]

In this communication a simple 3-step method for the preparation of highly active urease from jack-bean meal is described which involves an ethanol extraction with subsequent covalent chromatography followed by a gel filtration step.

The covalent chromatography procedure requires consecutive thiol–disulfide interchange reactions. Active urease, containing reactive thiol groups, is covalently bonded to the chromatographic material (agarose-2-pyridyl disulfide) *via* disulfide bridges and subsequently removed by a low molecular weight thiol compound (see Fig. 1a–b). Previous work with thiol–disulfide exchange immobilization of urease has been carried out using a gel containing 60 μmol SH groups per g of dried gel, 70 % of which could be activated for thiol-disulfide exchange by reaction with 2,2′-dipyridyl disulfide.[4]

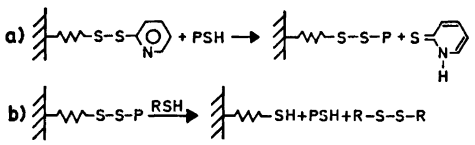

Fig. 1. Reaction scheme for covalent chromatography of jack-bean urease (PSH). RSH low molecular weight thiol such as DTT.

An attempt to increase the number of 2-pyridyl disulfide structures, and thus the immobilization capacity of the gel, by increasing its thiol content[7] was unsuccessful, since a 10-fold increase in gel bound –SH groups failed to increase the degree of activation.[8] The proposed explanation for this behaviour is the formation of disulfide bridges between neighbouring gel bound –SH groups by a thiol–disulfide exchange reaction involving newly formed 2-pyridyl disulfide residues and still unactivated thiol groups.[9] The effect is more pronounced with gels of high thiol concentration (> 100 μmol of SH-groups/g dried gel), since the thiol groups are then closely situated in space. The dipyridyl disulfide activation normally takes place in aqueous solution where the reagent is sparingly soluble (~1.5 mM).[5] An increased concentration of the activating agent, necessitating work in less than 100 % aqueous solution, should then diminish the mentioned undesirable side reaction. Indeed we have been able to show that the activation of highly substituted SH containing gel (600 μmol SH groups/g dried gel) in 60 % aqueous acetone, where the dipyridyl disulfide reagent is soluble enough to permit work at a concentration of 0.2 M, results in the conversion of *ca.* 67 % of the thiol groups into the active structure. This gives an agarose-2-pyridyl disulfide derivative containing about 400 μmol of 2-pyridyl disulfide structures/g dried derivative.

The increase in the amount of 2-pyridyl disulfide structures is particularly useful in work with raw extracts. Since they have undergone no selective treatment, they are bound to contain a sizeable amount of thiol containing compounds other than urease, leading to a competition with the enzyme for the reactive disulfide structures.

The release of gel-bound material is accomplished through reduction with dithiothreitol (Fig. 1b). This reaction, however, liberates not only thiol containing material of biological origin. Remaining active structures are simultaneously eliminated in the form of 2-thiopyridone.

The released material was passed through a column of Sephadex G-25 for elimination of low molecular weight material such as dithiothreitol and 2-thiopyridone. This step was introduced merely as a check on the performance of the covalent chromatography and should be omitted when the method is used preparatively.

Subsequent gel filtration on Sepharose 6B of void material from the previous step showed

that it contained about 50 % of high molecular weight material of insignificant urease activity. After removal of this, about 16 mg of urease with a specific activity of 2515 units/mg protein was obtained. This activity is about 50 % higher than the value reported previously for urease extensively purified by conventional techniques.[2] The overall efficiency of the preparation procedure is evident from Table 1, which indicates a 280-fold purification of the enzyme in the two steps following ethanol extraction of the jack bean meal. The capacity of the agarose-2-pyridyl disulfide derivative to bind urease active material was 5.1 mg/ml gel.

The pure urease preparation seemed to be quite stable, since no activity loss was detected when it was stored dissolved in 0.05 M Tris-HCl buffer containing 0.1 M KCl and 1 mM EDTA pH 7.2 at +4 °C for 3 weeks. To avoid aggregation and inactivation phenomena that might occur under longer periods of time, the active urease might be stored in immobilized form on the covalent chromatography gel. For some purposes it might even be preferable to use this immobilized conjugate which also shows urease activity.[4] In this form the urease should also be useful for studying its subunit organization which has not yet been fully elucidated despite considerable investigation.[10]

Materials and methods. Jack bean meal and dithiothreitol were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Sepharose 2B, Sepharose 6B and Sephadex G-25 were the products of Pharmacia Fine Chemicals Co., Uppsala, Sweden. 2,2′-Dipyridyl disulfide (2-PDS) was obtained from Aldrich-Europe (B-2340 Beerse, Belgium) and Nessler's reagent A (potassium tetraiodomercurate(II) solution) and Nessler's reagent B (sodium hydroxide solution) were obtained from E. Merck AG (Darmstadt, Germany).

Preparation of chromatographic material. A. Thiolation of agarose (Sepharose 2B) was performed according to Axén *et al.*[7] The thiolated agarose contained ~600 μmol of SH/g dry conjugate as determined by its reaction with 2,2′-dipyridyl disulfide.[5]

B. Activation of thiol-agarose. 25 g of freshly thiolated agarose (25 g of sedimented gel corresponds to 500 mg dry derivative) was washed with 0.025 M $NaHCO_3$ containing 60 % acetone and 1 mM EDTA and was then suspended in 20 ml of the same solution. The suspension was added to a solution of 2,2′-dipyridyl disulfide (2.6 g) in 20 ml 60 % acetone containing 0.015 % EDTA. The mixture was allowed to react under stirring for 1 h at +23 °C. The activated gel was then carefully washed free of excess reagent with 60 % acetone and 1 mM EDTA. The activated thiol-agarose contained 400 μmol of reactive disulfide structures per g dry conjugate. This figure which states the theoretical capacity of the chromatographic material to bind thiols, was determined by estimating the amount of 2-thiopyridone released as a result of reducing the activated gel with dithiothreitol.[5] The agarose-2-pyridyl disulfide derivative was stored as a suspension in 1 mM EDTA at +4 °C.

Preparation of jack bean meal extract. Jack-bean meal, 60 g, was mixed with 300 ml 0.05 M Tris-HCl buffer containing 36 % ethanol, 0.1 M KCl and 1 mM EDTA pH 7.2 (final pH was 6.5). The mixture was stirred for 5 min at +28 °C and was subsequently filtered on a Büchner funnel. The slightly turbid filtrate was then centrifuged (5000 *g*, 20 min). The supernatant (about 210 ml) was diluted to 300 ml by 0.05 M Tris-HCl buffer pH 7.2

Table 1. Efficiency of purification of urease from 50 g of jack-bean meal.

Procedure	Total activity [a] recovered (units)	Amount of dry weight material (mg)	Specific activity [a] (units/mg dry weight material)	Activity yield (%)
Ethanol extraction	56 100	6250 [b]	9	100
Covalent chromatography + gel filtration on G-25	48 000	32 [c]	1500	86
Gel filtration on Sepharose 6B	40 240	16 [c]	2515	72

[a] The ureolytic activity was determined essentially as described in Ref. 4. One unit is the amount of enzyme that liberates 1 μmol of NH_3 from urea at 25 °C in 1 min. [b] Calculated by dry weight determination. [c] Calculated from A_{280} using A_{280} (0.1 %) = 0.589 assuming that essentially all of the material is urease or polymers of urease.

(same as above) and the pH was adjusted to 7.2 by addition of 0.5 M NaOH. The final extract thus contained 25 % ethanol.

Covalent chromatography. A column with a total volume of 6.3 ml (1 × 8 cm) was prepared from the agarose-2-pyridyl disulfide gel. The column was equilibrated with 0.05 M Tris-HCl buffer pH 7.2 containing 0.1 M KCl and 1 mM EDTA. Jack bean meal extract (250 ml) was passed through the column at a flow rate of 20 ml/h. Ultraviolet absorption at 280 nm and ureolytic activity were determined in fractions of the effluent. Most of the UV-absorbing material passed through the column unretained. In the first 150 ml of eluate no urease activity was detected. A small activity that gradually increased to that of the sample applied was then observed within the next 100 ml of eluate. The column was then washed with Tris-HCl buffer (same as above) until A_{280} in the eluate was less than 0.04. Covalently bonded material was detached from the chromatographic material with 20 mM dithiothreitol (20 ml) dissolved in 0.05 M Tris-HCl buffer pH 8.0 containing 0.1 M KCl and 1 mM EDTA.

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Biosynthesis of a Quinol Glucoside in *Cornus*

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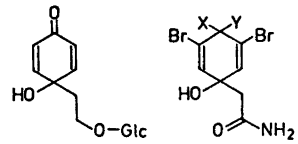
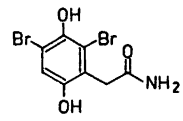
The glucoside *1*, first isolated from *Cornus foemina* Mill.,[1] but subsequently recognized as a component of probably all species of the *Cornus* subgenera Kraniopsis and Mesomora,[2] has no known obvious structural relative among constituents of higher plants, though the parent 4-alkyl-4-hydroxy-cyclohexa-2,5-dienone (quinol) system, or its formal addition or rearrangement derivatives, has been repeatedly encountered in natural products, such as *2*,[3] *3*,[4] *4*,[5] and *5*,[6] all from marine sponges of the genus *Verongia*; *6* (thelepin) from a marine worm;[7] and botrallin *7* of fungal origin.[8] An oxidative *in vivo* derivation of *1* from tyrosol *8*, possibly *via* salidroside *9*, a phenolic glucoside encountered within the families Crassulaceae, Ericaceae, Oleaceae, and Salicaceae, but also accompanying *1* in *C. foemina*[1] and other *Cornus* species,[2] appeared likely and has now been confirmed. We report the results.



1 *2* X,Y = =O *3* X = Y = OMe *4* X, or Y = OMe; Y, or X = OEt



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The potential precursors, L-tyrosine, tyrosol *8*, and salidroside *9*, labelled with ^{14}C or ^{3}H, were administered to young shoots of *C. stolonifera* Michx. After a metabolic period of 44 h, the glucosides *9* and *1* were isolated and purified by chromatography before their total radioactivities were determined. The results are presented in Table 1.

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