Lecithin

Hans Möllering and Hans-Ulrich Bergmeyer

Methods for the determination of phospholipids, in particular for lecithin (*e.g.* as measure of the egg content of foodstuffs), have so far proved rather unsatisfactory. In comparison with methods involving acid[1,2] or alkaline hydrolysis[3,4], the enzymatic determination of lecithin with lecithinase D[5–8] is preferable because of its greater specificity (refer, for example, to[9]).

Principle

Lecithinase (phospholipase) D catalyses the cleavage of choline from the lecithin molecule:

(1) $$\text{Lecithin} + H_2O \longrightarrow \text{phosphatidic acid} + \text{choline}$$

The reaction is activated by Ca^{2+} and ether. It proceeds quantitatively from left to right. Lecithin and phosphatidic acid are very soluble in ether, while choline is not. After the enzymatic reaction, phosphatidic acid can be separated from the choline by extracting with ether and the choline can be precipitated from the aqueous phase as the reineckate. The red-violet choline reineckate is soluble in acetone and the amount is measured colorimetrically at 520 mμ.

Reagents

1. Ether, A. R.
2. Acetic acid, A. R.
   should not reduce chromic acid.
3. Sodium acetate, A. R., anhydrous
4. Calcium chloride, A. R., $CaCl_2 \cdot 2\,H_2O$
5. Choline chloride, A. R.
6. Ammonium reineckate, A. R., $[Cr(NH_3)_2(SCN)_4]NH_4$
7. Acetone, A. R.
8. Chloroform, A. R.
9. Methanol, A. R.
10. Trichloroacetic acid, A. R.
11. Lecithinase D (phospholipase D)
    from cabbage leaves[9], lyophilized powder; commercial preparation, see p. 995.

Purity of the enzyme preparation

Lecithinase D isolated from white cabbage according to[9] is a relatively crude preparation, but it contains no enzymes which destroy choline. The enzyme should have a specific activity of *ca.* 0.5 units*)/mg. dry weight.

---

*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.[10]

1) *C. G. Daubney* and *G. E. W. Sexton*, Analyst *75*, 305 [1950].
2) *V. E. Munsey*, J. Ass. off. agric. Chemists *36*, 766 [1953].
3) *R. W. Engel*, J. Nutrit. *25*, 441 [1943].
4) *H. Salwin, M. D. Devine* and *J. H. Mitchell*, J. agric. Food Chem. *6*, 475 [1958].
5) *D. J. Hanahan* and *I. L. Chaikoff*, J. biol. Chemistry *169*, 699 [1947].
6) *M. Kates*, Canad. J. Biochem. Physiol. *33*, 575 [1955].
7) *E. Einset* and *W. L. Clark*, J. biol. Chemistry *231*, 703 [1958].
8) *F. M. Davidson* and *C. Long*, Biochem. J. *69*, 458 [1958].
9) *C. B. Casson* and *F. J. Griffin*, Analyst *84*, 281 [1959]; *86*, 544 [1961].
10) *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].

Preparation of Solutions (for *ca.* 20 determinations)

I. Acetate buffer (0.1 M; pH 5.6):
Mix 4.8 ml. 0.1 M acetic acid (5.8 ml. acetic acid made up to 1000 ml. with doubly distilled water) and 45.2 ml. 0.1 M sodium acetate solution (8.2 g. sodium acetate made up to 1000 ml. with doubly distilled water).

II. Calcium chloride (1 M):
Dissolve 14.7 g. $CaCl_2 \cdot 2\,H_2O$ in doubly distilled water and make up to 100 ml.

III. Ammonium reineckate solution (3% w/v):
Dissolve 600 mg. ammonium reineckate in methanol and make up to 20 ml.

IV. Choline chloride standard solution (2% w/v):
Dissolve 2 g. choline chloride in doubly distilled water and make up to 100 ml. Determine the content of the solution by titration with silver nitrate solution (formation of AgCl).

V. Trichloroacetic acid (3 M):
Dissolve 490 g. trichloroacetic acid in doubly distilled water and make up to 1000 ml.

VI. Lecithinase D (*ca.* 20 mg. protein/ml.):
Suspend 400 mg. lecithinase D in 20 ml. acetate buffer (solution I). Use the turbid enzyme suspension.

Stability of the solutions

Store the enzyme suspension at 0° C and prepare freshly each day; it rapidly loses activity. Also prepare the reineckate solution freshly each day. All other solutions are stable indefinitely at room temperature. However, care should be taken that no bacterial growth occurs.

Procedure

Experimental material

Analyse foodstuffs which contain large amounts of lecithin (*e.g.* eggs, egg liqueurs, milk powders, soya beans, mayonnaise) without preliminary treatment. Determine the dry weight of each sample.

Extract foodstuffs containing small amounts of lecithin (*e.g.* noodles, ice cream, cakes, pastry) in a Soxhlet apparatus with ether. All the lecithin is extracted into the ether layer. Analyse this.

Dissolve oils and fats in ether and precipitate the lecithin quantitatively with acetone (after addition of saturated $MgCl_2$ solution)[11]. Dissolve the precipitate in ether and analyse the solution.

For the preparation of foodstuffs for analysis, see also[12].

Extract animal tissues, blood, plasma and serum with ethanol-ether, petroleum ether-chloroform and hot chloroform-methanol (see[13,14]). Evaporate off the solvent and dissolve the residue in ether.

---

[11] *J. Nerking*, Biochem. Z. *23*, 262 [1910].
[12] *R. Kunze:* Lecithin. Rosenmeier & Dr. Saenger, Berlin 1941.
[13] *M. H. Hack*, J. biol. Chemistry *169*, 137 [1947].
[14] *R. G. Sinclair*, J. biol. Chemistry *174*, 343 [1948].

Enzymatic reaction

For each determination prepare a control without enzyme. This gives the choline content of the sample, while the experimental reaction mixture gives the choline + lecithin. Add to both reaction mixtures, either the solid sample finely powdered or a solution of the sample in ether (ether extract).

Pipette successively into 100 ml. volumetric flasks with ground-glass stoppers:

Experimental	*Control*
10.0 ml. acetate buffer (solution I)	11.0 ml. acetate buffer (solution I)
0.1 ml. $CaCl_2$ solution (II)	0.1 ml. $CaCl_2$ solution (II)
1.0 ml. enzyme suspension (VI)	
10.0 ml. ether extract of the sample	10.0 ml. ether extract of the sample
or	or
10.0 ml. ether and solid sample (equivalent to 5—50 mg. lecithin)	10.0 ml. ether and solid sample (equivalent to 5—50 mg. lecithin)

Shake vigorously on a mechanical shaker for 3 hours at room temperature. Into each flask pipette

1.00 ml. trichloroacetic acid (solution V),

mix, pour off and discard the ether phase. Extract the aqueous phase twice with 50 ml. portions of ether, pour off ether phase and discard. Filter the aqueous phase and analyse the filtrate.

Colorimetric measurements

Wavelength: 520 mμ; light path: 1 cm.; room temperature. Measure against pure acetone. Pipette into conical centrifuge tubes:

2.0 ml. filtrate
1.0 ml. ammonium reineckate solution (III)

Allow to stand for 3 hours in an ice bath. Centrifuge off the choline reineckate (red crystalline scales) and wash the precipitate twice with 3 ml. portions of doubly distilled water at 0°C. Dissolve the sediment in

3.0 ml. acetone

(the choline reineckate should dissolve completely) and centrifuge off insoluble material. Pour the clear supernatant into a cuvette and read the optical density.

Standard curve

Pipette into 100 ml. volumetric flasks:

10.0 ml. acetate buffer (solution I)
0.1 ml. $CaCl_2$ solution (II)
10.0 ml. ether
0.1—0.4 ml. choline standard solution (IV)
0.8—0.5 ml. water
1.0 ml. trichloroacetic acid (solution V).

Mix thoroughly, extract twice with 50 ml. portions of ether, discard the ether phase and filter the aqueous phase. Colorimetric measurements as described above. Plot the optical densities

(ordinate) against the mg. choline flask (abscissa). The amounts of choline taken per flask are 2, 4, 6 and 8 mg.

The standard curve cuts the abscissa at about 0.1 mg. because of the incomplete precipitation of the choline reineckate and the loss on washing with water. In spite of this the curve is reproducible.

Calculations

The optical density of the control (without enzyme) gives the free choline of the sample, while the optical density of the experimental flask (with enzyme) gives the free + liberated choline. The amount of lecithin is obtained from the difference of the optical densities ΔE.

Read off from the standard curve the mg. choline/flask corresponding to the ΔE. If 2 ml. of filtrate are used for the colorimetric measurements, then:

(mg. choline/flask) $\times$ 6 = mg. choline in the weight of sample taken (or in 10 ml. ether extract of the sample).

With an average molecular weight of 770 for lecithin, 1 mg. choline corresponds to 6.38 mg. lecithin (15.7%); therefore

(mg. choline/flask) $\times$ 6 $\times$ 6.38 = (mg. choline/flask) $\times$ 38.3 = mg. lecithin in the sample.

Example

200 mg. of whole-dried egg was weighed into the control and experimental flasks. The following optical densities were measured at 520 mμ; control: 0.006; experimental: 0.151; $\Delta E = 0.145$.
The value for $\Delta E = 0.145$ read off from the standard curve was 0.565 mg. choline/flask.

$0.565 \times 38.3 = 21.6$ mg. lecithin in 200 mg. sample
or 108 mg. lecithin/g. = 10.8% lecithin

Specificity

Triethanolamine is also precipitated as the reineckate; ethanolamine (colamine) and serine do not react under the conditions described here. Therefore kephalin is not estimated. Lecithinase D only hydrolyses the bond between the nitrogen base and the phosphoric acid[5]. According to[8] the enzyme reacts with the following lecithins: egg lecithin, hydrated egg lecithin, dipalmitoyl-L-α-lecithin, lysolecithin, synthetic dimyristoyl-L-α-lecithin, dipalmitoyl-DL-α-lecithin and distearoyl-DL-α-lecithin. Synthetic distearoyl-β-lecithin and sphingomyelin react slowly.