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[25] Bacterial Lipopolysaccharides

Extraction with Phenol–Water and Further Applications of the Procedure

By O. Westphal and K. Jann

Wander-Forschungsinstitut and Max-Planck-Institut für Immunbiologie, Freiburg-Zähringen, Germany

Introduction

Liquid phenol is known to be an excellent solvent for many proteins. The partition coefficient in biphasic phenol–water mixtures very often allows an almost complete extraction of proteins from aqueous solutions under controlled conditions of pH and ionic strength in a one-step operation. In contrast, polysaccharides, mucopolysaccharides, lipopolysaccharides, and nucleic acids are usually water-soluble but phenol-insoluble. Various polysaccharides can be precipitated from aqueous solution by adding liquid phenol (see, for example, *1*). Phenol is a weak acid, the dissociation constant at 18–19° in water being $1.1–1.2 \times 10^{-10}$ (*2*). Mixtures of phenol and water have a high dielectric constant. These facts form the basis of a method of partition of proteins and polysaccharides and/or nucleic acids between phenol and water. Separation of proteins from polysaccharides and nucleic acids by phenol–water is often effected by both the favorable partition coefficient and the dissociation power of phenol–water mixtures.

Morgan and Partridge (*3*) showed that diethylene glycol extracts (endotoxic or whole O-antigenic complex) of various Enterobacteriaceae, such as *Shigella dysenteriae* and *Salmonella typhosa*, are composed of specific polysaccharide, protein, and lipid material (see Vol. V [24]). In 90% liquid phenol solution, the whole complex dissociated. If the reaction mixture was dialyzed against water, the protein precipitated while the undegraded polysaccharide remained in the final water solution. The same method was applied by Goebel and co-workers (*4*) for the

Procedure

Procedure I (*9, 11, 15, 34*)

Gram-negative bacteria, after cultivation in suitable media, are centrifuged, and the sediment is washed with saline. The bacteria are killed by adding acetone and/or lyophilized from the frozen state.

Phenol–water Extraction

Twenty g. (dry weight) of bacteria, for example, Enterobacteriaceae (*Escherichia, Salmonella,* and so on), are suspended in 350 ml. of water at 65–68° (on a water bath); 350 ml. of 90% phenol, preheated to 65–68°, is then added with vigorous stirring, and the mixture is kept 10–15 min. at 65°. After cooling to about 10° by placing the vessel in an ice bath, the emulsion is centrifuged at 3000 rpm. for 30–45 min., which results in the formation of three layers: a water layer, a phenol layer, and an insoluble residue, the latter sometimes forming a layer at the phenol–water interphase. The water phase is sucked off, and the phenol layer and the insoluble residue are treated at 65–68° with another 350 ml. of water as described above. The combined water extracts are dialyzed 3–4 days against distilled water to remove phenol and small amounts of low-molecular-weight bacterial substances. The dialyzed, slightly opalescent solution, which contains the lipopolysaccharide and ribonucleic acid, is concentrated at 35–40° under reduced pressure to a volume of about 100 ml. After centrifugation for the removal of traces of insoluble material, the water solution is freeze-dried to give an almost white fluffy powder; yield 1.6–2.0 g. (8–10% of the dry weight of the bacteria). The crude extract is composed of about 40–50% of lipopolysaccharide (endotoxic O-antigen) and 50–60% of bacterial ribonucleic acid (RNA).

Removal of Nucleic Acid

The lyophilized crude extract is dissolved in water to give a 3% solution which is centrifuged for 6–8 hr. at 80,000 × g. The sediment is suspended in water, and the suspension is recentrifuged 2–3 times at 105,000 × g. for 3 hr. each. The final sediment is taken up in a minimum amount of water and freeze-dried; yield of bacterial lipopolysaccharide, 300–500 mg. (1.5–2.5% of the dry weight of the bacteria), containing 3% of nucleic acid.

It is known that polyanionic substances form water-insoluble salts with cationic detergents, such as cetyltrimethylammonium bromide ("cetavlon"). However, these salts dissolve in inorganic salt solutions, for example, sodium chloride, the solubility being dependent upon the

to precipitate the lipopolysaccharide, excess "cetavlon" remaining in solution. After standing 1–2 hr. at 0–4°, the precipitate is centrifuged and redissolved in water. After dialysis for 2 days against deionized water to remove sodium chloride, the solution is freeze-dried (Vol. V [17]); yield about 900 mg. of RNA-free lipopolysaccharide.

Procedure III

The lyophilized crude lipopolysaccharide–nucleic acid extract from the water phase of the phenol–water extraction (Procedure I) is dissolved in 0.5 *M* sodium chloride to give a 0.5–1% solution. A 2% solution of "cetavlon" in 0.5 *M* sodium chloride is added with stirring until the proportion of "cetavlon" to crude extract is about 1.5:1. The solution is now gradually diluted with water, and precipitates are collected by centrifugation as they appear. The RNA–"cetavlon" salt precipitates at a sodium chloride concentration of about 0.3 *M*. The final dilute solution is lyophilized (Vol. V [17]) and taken as the last fraction. The fractions are dissolved in 0.5 *M* sodium chloride and poured into a ten-fold volume of ethanol. After centrifugation, the sediment is dissolved in water, dialyzed, and freeze-dried (Vol. V [17]); yield of RNA-free lipopolysaccharide 30–40% of the crude extract.

Fractional "cetavlon" precipitation according to Procedure III proved to be of special value in cases, for example, in the *Salmonella* and *Escherichia* species, in which the water phase after phenol–water extraction, sometimes contained an additional acid mucopolysaccharide in addition to lipopolysaccharide and nucleic acid (*17a*).

Another means of obtaining nucleic acid-free bacterial lipopolysaccharide arose from the finding (*38*) that the phenol–water extraction of formalin-killed *Salmonella* bacteria gives a water phase containing lipopolysaccharide and only small amounts or no RNA. A further investigation (*39*) showed that bacterial RNA, after treatment of the bacteria with diluted formaldehyde (0.1–0.5%), is no longer extractable by phenol–water (Procedure I), probably because cross-linkages are formed between RNA and bacterial protein, giving rise to phenol–water-insoluble complexes. The formalin variation of the phenol–water extraction, however, needs to be investigated in more detail.

Further Applications of the Phenol–water Procedure

Partition of protein and polysaccharide or lipopolysaccharide between phenol and water can be applied for the dissociation and separation of specific precipitates of polysaccharide antigens with antibody (*40*). In principle, the precipitate is dispersed in water, and an equal volume of liquid phenol is added with stirring. After separation of the two phases