2-KETO-3-DEOXYGALACTONIC ACID AS A CONSTITUENT OF AN EXTRACELLULAR POLYSACCHARIDE OF AZOTOBACTER VINELANDII

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Received August 18, 1965

Most strains of Azotobacter vinelandii form extracellular polysaccharides under different cultural conditions. They are found as capsules or in the form of slime dissolved in the medium. Experiments concerning the chemical composition of such polysaccharides have been described by Kaufman (1960),Kaufman and Repaske(1960),and Cohen and Johnstone (1963,1964). Depending on the strain,glucose and rhamnose , glucose and hexuronic acids or glucose with rhamnose,sialic acid,galacturonic acid,mannuronolactone and o-acetyl groups were identified as constituents.

The following experiments describe for the first time 2-keto-3-deoxygalactonic acid as a glycosidically bound component of a polysaccharide.

METHODS

For polysaccharide production Azotobacter vinelandii 1484 was grown on a nitrogen free medium in liquid cultures with glucose as sole carbon source(Claus,1965). Extracellular polysaccharides were harvested at the end of the logarithmic growth phase. Because of the high viscosity of the cultures separation of the cells was accomplished only after a 75° heat treatment of the neutralized suspensions for 5 min during which the voluminous capsules of the cells were totally dissolved.

The polysaccharide was precipitated with a 2 % solution of cetyltrimethylammoniumbromide(Scott,1965),the precipitate was dissolved in 1 M NaCl and purified by repeated precipitations from 1 M NaCl solutions with ethanol. After dialysis

and lyophilization the polysaccharide showed a pale pink color. The yield was approximately 1 g per 1 of culture supernatant.

Paper chromatographic separations were carried out on Schleicher and Schüll 2043 b. The solvents used were A:ethylacetate-acetic acid-water 3-1-3(Jermyn and Isherwood,1949), B:ethylacetate-pyridine-water 5-2-5(Linskens,1959),C:phenol-water(Benson,1955) and D:pyridine-ethylacetate-acetic acid-water 5-5-1-3 (Fischer and Dörfel,1955). α-Keto acids were located and identified with o-phenylenediamine(Wieland and Fischer,1949),2-keto-3-deoxyhexonic acids with periodate-thiobarbituric acid(Warren,1960). The latter method was also used for deoxysugars.

2-Keto-3-deoxygluconic acid and 2-keto-3-deoxygalactonic acid were prepared from glucose or galactose(DeLey and Doudoroff,1957). Also both compounds were formed spontaneously from oxalacetic acid and glyceraldehyde under alkaline conditions after Ghalambor and Heath(1963). Preparative separation of the mixture of both keto acids was achieved by paper chromatography with phenol-water. 2-Keto-3-deoxygalactonic acid was decarboxylated to 2-deoxyxylose by means of ceric sulfate after reduction with $NaBH_4$. All other compounds were commercial preparations.

RESULTS

A detailed description of the formation and the chemical composition of the polysaccharide will be published elsewhere(Claus,1965). Analysis of a polysaccharide preparation showed a protein content of 2 %. The main constituent of the polymer was identified as L-rhamnose(64%).

Precipitation of the polysaccharide with cetyltrimethylammoniumbromide indicated that the polymer was acidic in nature. However,hexuronic acids normally found in bacterial polysaccharides could not be detected. As the main acidic constituent a compound was found which behaved like an α-keto acid and which formed ß-formylpyruvate after oxidation with periodate. The presence of sialic acids was eliminated by a negative Ehrlich reaction(Barry et al.,1963).

Attempts to fractionate the polymer electrophoretically were unsuccessful.Both of the main components - rhamnose and

keto acid - could not be separated in either Veronal buffer pH 9,0 or borate buffer pH 9,3.

The unhydrolyzed polysaccharide had no reducing properties(Somogyi-Nelson method;Neish,1952). During hydrolysis in 1 N sulfuric acid at 100° a complete release of the reducing compounds occured within 60 min. A hydrolysis curve with 2 maxima was obtained after the use of 0.1 N sulfuric acid. The first maximum was reached after 30 min ;the hydrolyzed compound amounted to nearly 30% of the total reducing capacity. Chromatographic tests demonstrated that at the time of the first maximum only keto acid was released from the polysaccharide. Free rhamnose was detected only after longer periods of hydrolysis. Keto acid was completely released within 60 min at 80° using 0.1 N sulfuric acid.

In contrast to the free acid,the keto acid bound to the polysaccharide was not destroyed by alkali(0.5 N NaOH,100°, 10 min);hydrolysis did not occur under these conditions. Reduction of the carbonyl group by means of sodium borohydride was impossible in the unhydrolyzed polymer,which may indicate that the keto acid was bound glycosidically to the polysaccharide. Moreover the release of the keto acid during hydrolysis before the appearance of free rhamnose suggested that the keto acid is located terminally in the polymer.

For the isolation of the keto acid 8 g of polysaccharide were dissolved in 760 ml water,heated to 80° and acidified with 40 ml 2 N sulfuric acid. After hydrolysis for one hour at 80° and neutralization with $Ba(OH)_2$ the hydrolysate was centrifuged. The cations in the supernatant solution were removed by Duolite C 3 and the keto acids were separated from the neutral sugar fraction by ion exchange using Duolite A 4. The keto acids were eluted with 0.5 N NH_4HCO_3 and NH_4^+ was removed by an excess of Duolite C 3. Finally the keto acid fraction was adsorbed on Dowex 1-formate(X 8,200-400 mesh). Solutions with increasing concentrations of formic acid(0.1-0.5 N) were passed through the column. The thiobarbituric acid reactive material was eluted in a nearly symmetrical peak with 0.25 N formic acid. After extraction with ether the total acid in the eluate amounted to 5.4 mmoles acid(as monocarboxylic acid). Determination of the semicarbazone,standardized against pyruvic acid,showed a

yield of 5.2 mmoles of keto acid. In the form of 2-keto-3-deoxyhexonic acid this value corresponds to approximately 12 % of the polysaccharide.

The chromatographically homogenous substance reacted with o-phenylenediamine giving an absorption maximum at 330 mμ, typical for α-keto acids (Lanning and Cohen, 1951). The semicarbazone gave a spectrum with a maximum at 250 mμ, also known for α-keto acids (MacGee and Doudoroff, 1954). The compound was decarboxylated in the presence of ceric sulfate (Meister, 1952) producing stoichiometric amounts of CO_2. Analysis of the isolated acid according to the periodate-thiobarbituric acid method (Waravdekar and Saslaw, 1959 ; Weissbach and Hurwitz, 1959) produced an alkali labile chromophor with an absorption maximum at 549 mμ. This is characteristic for ß-formylpyruvate which is formed from 2-keto-3-deoxyaldonic acids and related compounds. After reduction with $NaBH_4$ (Schenker, 1961) ß-formylpyruvate was no longer formed.

Determination of the periodate uptake (Levin and Raker, 1959) showed a consumption of 1,88 μmole periodate/μmole substrate. A theoretical consumption of 2 μmoles periodate was to be expected when 3 adjacent hydroxy groups were present in the carbon chain. The periodate oxidation produced a stoichiometric yield of formaldehyde, determined according to Speck (1962), which indicated a primary alcohol group on the terminal carbon atom. For this reason the unknown keto acid cannot be identical with either 4-deoxy-5-ketohexuronic acid described by Preiss and Ashwell (1962) or with 4-deoxy-5-ketoglucaric acid found by Dagley and Trudgill (1965). The formation of ß-formylpyruvate and formaldehyde and the simultaneous reduction of approximately 2 μmoles of periodate per μmole of keto acid strongly suggested that the unknown compound might be a 2-keto-3-deoxyhexonic acid.

To differentiate between the two possible epimeric 2-keto-3-deoxyhexonic acids, 2-keto-3-deoxygluconic acid and 2-keto-3-deoxygalactonic acid, the different rates of the release of ß-formylpyruvate from the keto acids by periodate was studied. The reaction rate is dependent upon the position of the diol groups on C 4 and C 5 (Waravdekar and Saslaw, 1959; Preiss and Ashwell, 1962). The congruence of the re-

action rates of both the keto acid from the polysaccharide and the synthetic 2-keto-3-deoxygalactonic acid is shown in Fig.1.

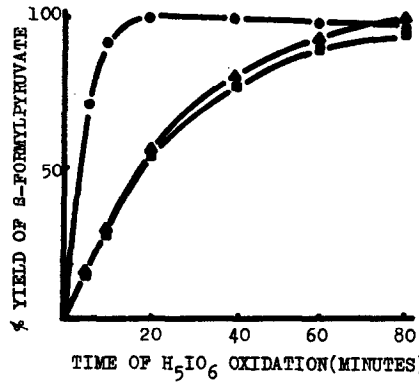

Fig.1. Rate of release of ß-formylpyruvate upon periodate oxidation of 0.04 µmoles of 2-keto-3-deoxygluconic acid(●), 2-keto-3-deoxygalactonic acid(▲)and the keto acid isolated from the polysaccharide(■).

In different solvent systems the polysaccharide keto acid exhibited the same R_f-values as synthetic 2-keto-3-deoxygalactonic acid. The separation of the two epimeric keto acids could be accomplished only in solvent C.

An additional confirmation of the identity of the keto acid was obtained by oxidative decarboxylation of the previously reduced keto acid to the corresponding 2-deoxyaldose. With periodate-thiobarbituric acid the degradation product showed an absorption maximum at 532 mu typical for 2-deoxyaldoses (Waravdekar and Saslaw,1959). After reaction with sulfuric acid - cysteine (Dische,1962) the decarboxylated compound exhibited an absorption spectrum characteristic for 2-deoxypentoses. The 2-deoxypentose behaved chromatographically like 2-deoxyxylose (Tab.1).

Tab. 1
Paper chromatography of the decarboxylated keto acid

Compounds	R_f in Solvent Systems	
	A	B
2-deoxyribose	0,45	0,46
2-deoxyxylose	0,48	0,53
decarboxylated keto acid	0,48	0,53

The rate of release of malondialdehyde from the decarboxylated keto acid by periodate treatment again showed a cis-trans arrangement of the diol groups now on C 3 and C 4 as present in 2-deoxyxylose. Since 2-deoxyxylose is the decarboxylation product of 2-keto-3-deoxygalactonic acid it was confirmed that the compound isolated from the poly - saccharide is identical with this keto acid.

DISCUSSION

Polysaccharides of microorganisms often contain as acidic components hexuronic acids in concentrations up to 50%. Recently other acidic constituents have been shown such as pyruvic acid (Orentas et al.,1963),sialic acids (Dewitt and Rowe,1961) and ketodeoxyoctonic acid (Heath and Ghalambor,1963). It was found in the described experiments that Azotobacter vinelandii forms 2-keto-3-deoxygalactonic acid as a main constituent of the capsule polysaccharide. Preliminary tests suggest that also other strains of this bacterium freshly isolated from soil may contain the same keto acid in the capsule polysaccharide in concentrations up to 20%.

Nothing is known about the pathway for the formation of ketodeoxygalactonic acid by Azotobacter vinelandii. Until now this acid has only been described as an intermediate in the metabolism of galactose and galactonic acid in Pseudomonas saccharophila and Gluconobacter liquefaciens(DeLey and Doudoroff,1957,Stouthamer,1961). Since in experiments described here glucose was used as sole carbon source it is

more probable that the keto acid is formed in a synthetic process, especially since the compound was also detected when cells were grown on benzoate or acetate. Experiments on the metabolic pathway of the keto acid formation are in progress.

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