Identification of 2-Oximino Acids in Yeast by GC – MS

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In view of its toxic nature and reactivity with carbonyl compounds, hydroxylamine (HA) is most likely to be found in combined form, especially as oximes. Formation of α-keto acid oximes (2-oximino acids) has been suggested as a way for microorganisms to utilize HA as the sole source of nitrogen. Oxime formation could be a pure chemical process and/or an enzymatic one. Free HA has mutagenic properties even in extracellular concentrations of 10⁻⁵ M. However, the yeast Endomycopsis lipolytica has the ability to grow on HA-concentrations as high as 4 x 10⁻² M. This yeast displays a high cellular content of α-keto acids with predominance of 2-oxoglutaric acid (2-oxopentanedioic acid).

In previous investigations only the oxime of pyruvic acid (2-oximinopropionic acid) was found in microorganisms grown on incompletely reduced nitrogen. Pyruvic acid oxime was here converted into acetonitrile with subsequent gas liquid chromatographic (GLC) analysis.

In this study GLC – MS has been used in order to improve the analytical procedure for determining the oximes of glyoxylic (GOAO) (2-oximinooctanoic acid), pyruvic (PYAO) (2-oximinopropionic acid), oxalacetic (OAAO) (2-oximinobutanoic acid), and 2-oxoglutaric acid (OGAO) (2-oximinopentanedioic acid) in E. lipolytica.

GLC of cell extract of E. lipolytica (Fig. 1) revealed the presence of PYAO and OGAO. GOAO could not be detected at all while small amounts [<2 μg (g⁻¹ dry wt)] of OAAO could be traced when a large amount of cell mass was used. However the presence of this oxime could not be confirmed by MS. GLC – MS analyses gave no information about geometric isomers. OAAO, however, is reported to yield two isomers.

Maximum levels of PYAO and OGAO were in the range of 65 – 75 μg (g⁻¹ dry wt) for cells in late log phase. At the same stage of growth the levels of pyruvic acid and 2-oxoglutaric acid were 40 and 150 μg (g⁻¹ dry wt), respectively. The low content of OGAO in relation to the high amount of 2-oxoglutaric acid is notable. This could imply a fast turnover rate for OGAO or instability of this oxime at conditions prevailing in the cells. The oxime group of OGAO could also be transferred to e.g. pyruvic acid by means of transoxinases. A non-enzymatic transfer of oxime groups to keto acids was not observed in the present study.

Cryptococcus albidus, a yeast with the ability to grow on nitrate, contained PYAO and OGAO at a lower level than in E. lipolytica. These two yeasts together with Saccharomyces cerevisiae displayed no oximes when grown on ammonia, indicating the significance of oximes in the reductive assimilation of nitrogen.

Experimental. Extraction. Cells of E. lipolytica were grown in a glycerol medium with HA as the sole nitrogen source at a concentration of 8 x 10⁻³ M. A cell mass corresponding to about 1 g cell dry weight was harvested by centrifugation, washed, suspended in 20 ml pyridine: H₂O (4:1 v/v), and homogenized. The pyridine phase was evaporated and the residue was dissolved in 0.7 ml cold water, saturated with NaCl, and acidified to pH 2. The oximes were extracted four times with 0.6 ml cold ethyl acetate. Diethyl ether and acetone were also
Table 1. Recoveries of α-keto acid oximes from test solution ranging from 25 to 100 µg of each oxime.

<table>
<thead>
<tr>
<th>Oxime</th>
<th>Average recovery %</th>
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<tbody>
<tr>
<td></td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>GOAO</td>
<td>90</td>
</tr>
<tr>
<td>PYAO</td>
<td>95</td>
</tr>
<tr>
<td>OAAO</td>
<td>60</td>
</tr>
<tr>
<td>OGAO</td>
<td>85</td>
</tr>
</tbody>
</table>

tested as extraction solvents but, as shown in Table 1, these solvents were found to yield a lower recovery in comparison with ethyl acetate. Moreover, when applied to cell material, acetone extraction resulted in a complicated gas chromatogram with many unresolved peaks.

Trimethylsilylation. The combined ethyl acetate extracts (2.4 ml) were evaporated to dryness and the residue silylated with 100 µl BSTFA containing 1% TMCS. Optimal conditions were obtained when the mixture was warmed at 50°C for 1 h. Later experiments have shown that silylation for 10 min at room temperature with a mixture of 50 µl pyridine and 50 µl BSTFA:TMCS gave comparable silylation effect.

GLC apparatus. The trimethylsilylated oximes were separated on a Perkin Elmer 3920 instrument and detected by a flame ionization detector. The injections were performed via a Grob-type split injector block and the GLC columns used were either OV 101 or SE 30 WCOT glass capillary columns, 25 m long and 0.2 mm i.d. Nitrogen was used as carrier gas with a flow rate of 1.0 ml/min and a split ratio of 1:50. The temperature program was 60 - 250°C at 8°C/min.

GLC - MS apparatus. The GLC - MS system used was a combined Carlo Erba Fractovap 2101 gas chromatograph and a Varian MAT 112 mass spectrometer coupled to a Spectro system 100 MS computer. The GLC conditions were the same as above except that helium was used as carrier gas at 2 ml/min. Ionization was performed in EI mode at an IP of 70 eV, emission current of 1500 µA, and an accelerating voltage of 0.8 kV. High resolution MS was performed on reference substances with an AEI, MS 902 instrument operating in EI mode at 70 eV, 200 µA, and 8 kV via a direct inlet probe.

Gas chromatograms. In a reference mixture the four α-keto acid oximes used in this study are well separated under the conditions described above. Fig. 1 shows a typical gas chromatogram of a TMS-derivatized extract from E. lipolytica where the PYAO and OGAO fractions are seen. GOAO and OAAO are to be expected at fractions 1 and 2, respectively.

Mass spectra. The identity of the four α-keto acid oximes in the cell extracts were determined by high and low resolution mass spectrometry by using reference substances. The diagnostically important ions for these substances were the nitrogen-containing ions e.g. M and M - 15. Other ions useful in the identification of the monocarboxylic oximes include M - 43 and a fragment derived from PYAO due to loss of - COOSi(CH₃)₃.12 The latter fragment was dominant in the spectra of the diacids oximes.13 OGAO also has a prominent ion at m/z 170 that originates through loss of [(CH₃)₃Si]⁻ and - COOSi(CH₃)₃ from the molecular ion. Other prominent ions (nondiagnostic) typical for silylated compounds were m/z 73 [(CH₃)₃Si]⁺ and m/z 147 [(CH₃)₃Si-O-Si(CH₃)₂]⁺.

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