Mechanism of Formation of Chloropropanols Present in Protein Hydrolysates

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Chloropropanols are formed in protein hydrolysates by the reaction of hydrochloric acid with residual lipids associated with the proteinaceous materials used in their production. The products formed from glycerol, triolein, 1,2-diacyl-sn-glycero-3-phosphorylcholine and soya meal have been analyzed by thin-layer and gas chromatography. The yields and isomer ratios of the chloropropanols and dichloropropanols formed are interpreted in terms of reaction mechanisms for their formation, which involve preferential nucleophilic substitution by the chloride anion at positions activated by neighboring ester groups. These provide anchimeric assistance and govern regioselectivity through steric and electronic effects.

KEY WORDS: Chloropropanols, glycerides, glycerol, hydrochloric acid, nucleophilic substitution, phospholipids, protein hydrolysates.

Protein hydrolysates are widely used as seasonings and ingredients in processed savory foods products. They are commonly produced by hydrochloric acid hydrolysis of proteinaceous by-products from edible oil extraction, such as soybean meal, rapeseed meal and maize gluten. Studies by Velisek et al. (1–6) have demonstrated the presence in protein hydrolysates of several chloropropanols and their fatty acid esters, and their formation in model hydrolysis systems from lipids (both synthetic and residual lipids from raw materials used in hydrolysate manufacture). The main chloropropanol detected by Velisek in protein hydrolysates was 1,3-dichloropropan-2-ol (1,3DCP), together with smaller amounts of 2,3-dichloropropan-1-ol (2,3DCP) and 3-chloropropanol (4,6). Model studies with lipids (3) and glycerol (2) strongly suggest that 3-chloropropan-1,2-diol (3MCPD) and 2-chloropropan-1,3-diol (2MCPD) may also be expected in commercial hydrolysates, but to date no methods for their determination or data on their levels in protein hydrolysates have been published.

In a recent publication (7) we report methods of analysis suitable for the confirmed determination of a range of chloropropanols in protein hydrolysates and derived food products. The methods permit the quantitative determination of 3MCPD, 2MCPD, 1,3DCP and 2,3DCP, the principal chloropropanols found in traditionally processed hydrolysates. Using these analytical methods we have investigated the mechanisms by which these chloropropanols are formed from glycerol, its esters and soybean meal.

MATERIALS AND METHODS

Materials. Anhydrous sodium sulphate and diethyl ether were analytical reagent (AR) grade from Fisons (Poole, U.K.), while sodium hydroxide and sodium bicarbonate (AR grade) were obtained from BDH (Poole, U.K.). Extrelut 20 columns were obtained from Merck (Darmstadt, Germany). Heptafluorobutyryl imidazole (HFBI) was obtained from Pierce (Oud-Beijerland, The Netherlands), hexane from Rathburn Chemicals Ltd. (Walkerburn, Scotland), 3-chloropropanol-1,2-diol (calibration standard) from Fluka (Buchs, Switzerland), 1,3-dichloropropan-2-ol (1,3DCP) and 2,3-dichloropropan-1-ol (2,3DCP) from Paltz & Bauer (Stamford, CT), p-dichlorobenzene (PCB) from Aldrich (Eillingham, U.K.), glycerol-d5 (penta-deuterated) from Merck Sharpe & Dohm Isotopes, and oleic acid and trilin from Sigma Chemical Co. (St. Louis, MO). 1,2-Diacylphosphorylcholine (PC) was purified from soya lecithin and 2-chloropropan-1,3-diol was provided by Dr. F. Ruf (Maizena GmbH, Heilbronn, Germany).

Hydrolysis reactions. Unless stated differently elsewhere, reaction conditions were as follows: Substrate (1 × 10⁻⁴ moles) was weighed into a 2-mL septum-capped glass vial. Hydrochloric acid (1 mL, 5.5 M) was added and the vial was capped, shaken and heated at 107°C for 16 hr.

Sample preparation. The reaction vial containing the reaction mixture was cooled to ambient temperature and uncapped, and its contents were quantitatively transferred, by using distilled water, to a graduated test tube (25 mL). Each sample was adjusted to 20 mL and neutralized by the addition of distilled water and sodium hydroxide solution (40% w/v). The neutralized solution was applied to an Extrelut 20 column (Merck) and allowed to equilibrate for 15 min (Note: viscous samples may be mixed with Extrelut refill material before it is packed into a column). Chloropropanols were recovered from Extrelut columns as follows: For analysis of all chloropropanols (mono-ols and diols), a two-stage elution separating the less abundant mono-ols from the more abundant diols and other interfering compounds proved necessary. The Extrelut column was first eluted with hexanediehtyl ether (90:10, v/v) to collect 50 mL (eluate 1) containing the mono-ols, followed by diethyl ether to collect 250 mL (eluate 2) containing the chloropropanodiols. For analysis only of the chloropropanodiols the Extrelut column was eluted simply with diethyl ether to collect 250 mL (eluate 3).

Derivatization. Eluate 1 (5 mL), or eluate 2 or 3 (1 mL), or the calibration solution (1 mL) was pipetted into a 25-mL volumetric flask. PDCB standard solution (1 mL) was made up to 25 mL with hexane. HFBI (200 μL) was added, the solution was mixed and allowed to stand at ambient temperature (20°C) for 15 min with intermittent shaking.

The mixture was then transferred to a screw-capped vial containing distilled water (2 mL) and shaken (1 min), the separated organic layer was washed twice more, and a 10-mL aliquot was filtered through a 4-cm column of anhydrous sodium sulphate (4 g) before analysis by gas chromatography (GC). Dried derivatized solutions were found to be stable for up to three days under refrigeration. When necessary, limits of determination were improved by taking a larger aliquot of the appropriate Extrelut fraction for derivatization. When aliquots greater than 2 mL are taken, ether eluates 2 and 3 must be blown dry under a gentle stream of nitrogen before derivatization to overcome sporadic ether-based interference problems.

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Individual chloropropanols were quantitated by analysis with a capillary gas chromatograph and an electron capture detector (GC-ECD), while total chloropropanediols were quantitated by packed-column gas chromatography and an ECD.

Analysis by GC-ECD. Heptafluorobutyrate esters of the chloropropanols were separated and determined by gas chromatography with electron capture detection, using a Perkin Elmer 8320 (Perkin Elmer, Norwalk, CT) or a Philips PU 4550 gas chromatograph (Phillips, Cincinnati, OH) fitted with a splitless injection port. Operating conditions were optimized as follows: Column: 25 m × 0.2 mm fused silica with 0.33-μm film of immobilized OV 1 (part No. 19091Z-1020, Hewlett Packard, Palo Alto, CA). Oven temperature program: 50°C for 2 min, 50°C to 85°C at 1°C/min, 85–250°C at 20°C/min, hold at 250°C for 20 min. Helium carrier gas: 12 psig. Injector: Splitless at 280°C, vent opened after 0.6 min. Injection volume: Up to 3 μL. EC detector temperature: 350°C (Perkin Elmer 8320). Detector responses to derivatized chloropropanols and the PDCB internal standard vary with temperature in an instrument-dependent fashion. The ECD should be operated within a temperature range over which detector response is least affected by temperature change.

Analysis by packed column GC. A Pye Unicam (Cambridge, U.K.) 304 gas chromatograph fitted with an ECD and operating with an injection temperature of 140°C and oven temperature of 125°C (isothermal) was used. Separation was carried out on 10% SP-2250 supported on 100/120 Supelcoport packed in a 5 ft × 0.4 cm column; nitrogen was used as the carrier gas at 30 mL/min.

The combined chloropropanadiol peak had a 1.9-min retention time and was quantitated by comparison of the peak height with that of a calibration graph. The calibration graph was constructed from the peak heights of a range of standard solutions of 3MCPD prepared in ether and derivatized alongside the sample solutions.

Thin-layer chromatography. Thin-layer chromatographic analysis was carried out on precoated silica plates; solvent mixtures used for development were chloroform/methanol/acetic acid/water (85:15:10:3, v/v) and petroleum ether/formic acid (80:20:1, v/v). Spots were visualized by spraying with sulphuric acid and heating at 110°C.

RESULTS

Although it has been established (1–6) that lipids are major precursors to chloropropanols, previous studies have not ruled out carbohydrates as precursors. Pentosan (arabinoxylans) and pectin (mainly methyl-esterified galacturonic acid) react with hydrochloric acid to produce substantially lower yields of chloropropanediols than obtainable from crude soya meal or maize gluten, indicating that they are not the principal precursors (Table 1). Maize glutens which have been extracted with chloroform/methanol (2:1) (to reduce the lipid content), react with hydrochloric acid to generate a lower yield of chloropropanols than nonextracted maize gluten. Hydrolysis of a mixture of extracted maize gluten and glycerol (equivalent to the level of glycerol present as glycerol esters) led to a lower (40%) yield of chloropropanols than obtained from crude maize gluten, suggesting that glycerol esters rather than glycerol are the precursors to chloropropanols.

Crude proteins used for the manufacture of hydrolysates contain a range of glycerolipids and phospholipids. Triolein and 1,2-diacyl-sn-glycerophosphorylcholine were used in this investigation as model substrates to represent these classes of materials. Equimolar amounts of triolein, 1,2-diacyl-sn-glycerophosphorylcholine (PC) and glycerol were treated with hydrochloric acid under conditions typical of hydrolysate manufacture, and yields of chloropropanols were determined (Table 2). Yields of chloropropanol from triolein and PC were significantly higher than from glycerol (2.6 times and 1.8 times, respectively). Triolein gave a much higher 3MCPD/2MCPD ratio than that obtained from glycerol and PC, suggesting that the three substrates react by different mechanisms to form chloropropanols.

Hydrolysis of mixtures of glycerol (pentadeutero, d5)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate/HCl</th>
<th>HCl (molarity)</th>
<th>Reaction conditions</th>
<th>Total chloropropanodiols (μg/g starting material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya meal</td>
<td>1:10</td>
<td>5.5M</td>
<td>Reflux/11 hr</td>
<td>235</td>
</tr>
<tr>
<td>Soluble pentosan</td>
<td>1:10</td>
<td>5.5M</td>
<td>Reflux/11 hr</td>
<td>5</td>
</tr>
<tr>
<td>DM 33.7 pectin</td>
<td>1:15</td>
<td>5.5M</td>
<td>Reflux/11 hr</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total chloropropanoils</th>
<th>Yield relative to glycerol</th>
<th>3 MCPD/2MCPD ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>2.2%</td>
<td>1</td>
<td>2.7</td>
</tr>
<tr>
<td>Triolein</td>
<td>5.7%</td>
<td>2.6</td>
<td>10.1</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>4.0%</td>
<td>1.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

1Pentadeuterochloropropanoic acid could be resolved from chloropropanoic acid by capillary gas chromatography.