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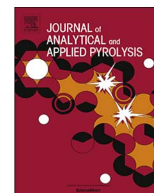

Analytical pyrolysis of ovalbumin

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ABSTRACT

In this study the thermal degradation of ovalbumin (OVA) under nitrogen atmosphere was investigated. For this scope, a multi instrumental approach based on thermogravimetry (TG), thermogravimetry coupled with infrared spectroscopy (TG/FTIR) and pyrolysis coupled with mass spectrometric detection, i.e. flash pyrolysis-coupled with gas chromatography-mass spectrometry (Py/GC/MS), evolved gas analysis coupled with mass spectrometry (EGA/MS) and double shot pyrolysis-coupled with gas chromatography-mass spectrometry (DSP/GC/MS), was used. The pyrolysis of a protein involves a combination of several complex mechanisms resulting in a very high number of products. The study highlighted that pyrolysis of OVA produces low-molecular weight gasses, such as CO_2, H_2O, HCNO, NH_3 and CO, as main compounds. In addition, a series of organic compounds containing heteroatoms and unsaturations were also identified, whose formation occurred at different temperatures over the pyrolytic process. Among these, cyclic pyrolysis products were identified: dialkyl substituted 2,5-diketopiperazines (DKPs) and, for the first time, unsaturated-DKPs (un-DKPs), 3,5-alkyl-3,4-dihydro-2H-pyrrole-2,4-diones (ADPDs) and 3-alkenyl-5-alkyl-pyrrolidine-2,4-diones (AAPDs). These compounds are formed below 350°, and are produced by cyclisation reactions of two neighbouring amino acids. Pyroglutamic acid was also found among the main pyrolysis products of OVA, obtained as pyrolytic product of Glu, which is the most abundant amino acid in OVA. Aromatic compounds, such as pyridine, pyrrole, toluene, alkyl-benzenes and alkyl-pyrroles, phenol and alkyl-phenols, benzeneacetonitrile, benzenepropanenitrile, indole and alkyl-indoles, were detected, produced over a wide range of temperatures. This study highlighted for the first time that aromatic compounds produced below 320 °C are associated to the pyrolysis of specific amino acid side chains, while at higher temperatures, they are the pyrolysis products of the residual material remaining after condensation reactions, pyrolytic scissions and cyclization reactions.

1. Introduction

Amino acids [1–3], polypeptides [4], proteins and proteinaceous materials [5–7] have been extensively investigated by analytical pyrolysis. The pyrolitic profiles of these materials are very hard to interpret as they comprise extremely complex and mostly unresolved mixtures of different classes of organic compounds, mainly containing unsaturations and heteroatoms.

The primary path of the thermal degradation of amino acids involves dehydration, decarboxylation and deamination reactions [8–10]. Moreover, condensation and dehydration reactions of two amino acids lead the formation of cyclic pyrolysis products, such as dialkyl substituted 2,5-diketopiperazines (DKPs)[10]. The pyrolytic profile of single amino acids is strongly influenced by the reactivity of their side chains [7–9]. As a consequence, specific compounds can be obtained from each amino acid, such as maleimide and succinimide from Asn, butanenitrile and derivatives from Leu and Ile, toluene and ethylbenzene from Phe, indole from Trp, phenol from Tyr and pyrrole from Hyp and Pro [7]. Following these primary processes, a significant number of products arise from secondary reactions, such as nitriles and amines [8,9].

DKPs are relevant pyrolysis products not only of amino acids, but also of peptides, polypeptides and proteins. They sublime at 120–215 °C and their formation occurs via thermal cyclization of two neighbouring amino acids in the polypeptide chain, followed by loss of water [4,8,9,11–13]. Studies on different dipeptides have demonstrated that several factors, including the steric and electronic nature of the substituents, have relevant effects on the formation of DKPs [14,15]. It was demonstrated that, given Gly at the N-terminus of a dipeptide, the amount of DKPs produced varies as a function of the chemical nature of the side chain of the amino acid at the C-terminus. Sulphur, nitrogen and oxygen atoms (Ser, Thr and Cys) significantly affect the polarization of the C-terminal carbonyl bond, rendering it more susceptible to nucleophilic attack, and thus promoting the formation of DKPs [14].

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For the same reasons, the cyclic substituent of Pro shows a favourable reactivity to form DKPs upon pyrolysis, while the presence of a carboxylic acid in the side chain inhibits thermal cyclization reaction, and Asp and Glu do not form DKPs. Dipeptides containing Lys, Arg, Asn and Gln also show a minimal tendency to form DKPs. The steric hindrance of the substituents in aliphatic amino acids also affects the formation of DKPs: Ala gives the highest amount of DKPs, Ile and Leu give lower yields in DKPs formation, while the isopropyl group of Val significantly decreases the yield of cyclisation reactions [14].

The thermal decomposition pathways of polypeptides and proteins are more complex than those of dipeptides and involve a higher number of reactions [11,16–18]. The main pyrolysis products are simple volatile compounds, such as CO_2, H_2O, NH_3 and CO, followed by, to a lower extent, a variety of organic compounds, often containing heteroatoms and unsaturations [7,12]. Aromatic compounds, such as toluene, pyrrole, benzenepropanenitrile, phenol and indole dominate the pyrograms of these materials and they are often associated to the pyrolysis of specific amino acids [7,12,19]. Pyrolysis of peptides and proteins also leads to the formation of cyclic saturated and unsaturated compounds, deriving from the cyclization of two neighbouring amino acids. Among these, DKPs are the main cyclization products [13], and their composition reflects the protein sequence and the reactivity of the side chains of most abundant amino acids. The formation of other cyclic compounds, such as imidazolindiones and pyrrolidin-diones have been evidenced in some proteins [7,11,20,21].

This paper investigates the thermal degradation of ovalbumin (OVA) in inert atmosphere. Few previous studies have been published, highlighting the formation of aromatic compounds, such as indole, benzyl nitrile, benzyl propan nitrile and 4-methyl phenol, as well as DKPs [7,19,22–24]. The fraction of DKPs is dominated by Pro-Leu, Pro-Ile and a minor amount of Pro-Val [22,24]. Pyroglutamic acid is also observed in the chromatogram of OVA, as a cyclization product of Glu, one of the most abundant amino acid in the protein [22]. A recent study on the characterization of the most common proteinaceous paint binders, such as casein from milk, egg white and animal glue, highlighted that alkyl-substituted 2,5-diketopiperazines (DKPs) are produced at lower temperatures, while at higher temperature the molecular pattern of the products of thermal degradation is dominated by aromatic compounds containing heteroatoms [24].

In this work a multi instrumental approach was used, based on thermogravimetric analysis (TG), thermogravimetric analysis coupled with infrared spectroscopy (TG/FTIR) and pyrolysis coupled with mass spectrometric detection, such as flash pyrolysis-coupled with gas chromatography-mass spectrometry (Py/GC/MS), evolved gas analysis coupled with mass spectrometry (EGA/MS) and double shot pyrolysis-coupled with gas chromatography-mass spectrometry (DSP/GC/MS). The study allowed to identify the main pyrolysis products of OVA and gave insight into the main thermal degradation mechanisms taking place at different temperatures.

2. Material and methods

2.1. Material

Ovalbumin (OVA, chicken egg, grade VI, EC 232.692.7) was purchased from Sigma Aldrich and used without further purification for all the experiments.

2.2. Thermogravimetric analysis (TG and TG/FTIR)

A TA Instruments Thermobalance model Q5000IR equipped with an FTIR (Agilent Technologies) spectrophotometer Cary 640 model for evolved gas analysis (EGA) was used. TG-FTIR measurements were performed in the temperature range 40–900 °C at a rate of 20 °C/min under nitrogen flow (90 mL min^{-1}), in the range 600–3000 cm^{-1} with a 4 cm^{-1} width slit. To reduce the strong background absorption from water and carbon dioxide in the atmosphere, the optical bench was purged with nitrogen. A background spectrum was taken before each analysis in order to zero the signal in the gas cell and to eliminate the contribution of ambient water and carbon dioxide. The amount of sample used for TG-FTIR measurement was of about 3 mg. Data were collected using Agilent Resolution Pro version 5.2.0.

2.3. Pyrolysis coupled with mass spectrometric detection

A micro-furnace Multi-Shot Pyrolyzer EGA/Py-3030D (Frontier Lab), coupled on-line with gas chromatograph 6890 Agilent Technologies (USA) with an Agilent 5973 Mass Selective Detector was used.

2.3.1. Instrumental set-up

2.3.1.1. Pyrolyser. Py/GC/MS: the temperature of the furnace was set at 550 °C (for 60 s) and the interface temperature was 180 °C.

DSP/GC/MS: in the first pyrolysis step, the temperature of the micro-furnace was kept at 330 °C for 60 s, while in the second step at 600 °C for 20 s.

EGA/MS: the temperature of the micro-furnace was ramped from 50 °C up to 700 °C (10 °C/min) and the interface temperature was 180 °C.

2.3.1.2. Gas chromatograph mass spectrometer. Py/GC/MS and DSP/GC/MS: The gas chromatograph was equipped with an HP-5MS fused silica capillary column (stationary phase 5% diphenyl–95% dimethyl-polysiloxane, 30 m × 0.25 mm i.d., Hewlett Packard, USA) and with a deactivated silica pre-column (2 m × 0.32 mm i.d., Agilent J&W, USA). Chromatographic conditions were as follows: initial temperature 40 °C, 2 min isothermal;10 °C/min up to 140 °C; 6 °C/min up to 280 °C; 10 C/min up to 300 C, 30 min isothermal. Carrier gas: He (purity 99.995%), constant flow 1.2 ml/min. The split/splitless injector was used with at 1:10 split ratio. The inlet temperature was 280 °C, and the MS transfer line at 300 °C. The mass spectrometer was operated in EI positive mode (70 eV, scanning m/z 50–600).

EGA/MS: The gas chromatograph was equipped with a deactivated and uncoated stainless steel transfer tube (UADTM-2.5N, 0.15 mm i.d. × 2.5 m length, Frontier Lab). Carrier gas: He (purity 99.995%), constant flow 1.2 mL/min. The split/splitless injector was used with at 1:20 split ratio. The inlet temperature was kept at 280 °C, the chromatographic oven at 300 °C and the MS transfer line at 300 °C. The micro-furnace interface temperature was kept at 100 °C higher than the furnace temperature until the maximum value of 300 °C. The mass spectrometer was operated in EI positive mode (70 eV, scanning m/z 50–600).

2.3.2. Sample handling

The sample (50 μg) was placed into a stainless steel cup, then inserted into the micro-furnace and the evolved gasses are transferred into the GC injection port through a flow of He. Stainless steel cups, after use were emptied and flame cleaned. A blank measurement was applied before the analysis of each sample, in order to verify the absence of contamination in the cup. Samples were analysed in triplicates. The relative standard deviations (RSD) of the areas of chromatographic peaks and their relative retention times were below 20% and 1%, respectively, as discussed more in detail in a previous study [24]

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