Advances in Chemistry of Isothiocyanate-derived Colourants

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Abstract: This study is focused on the reactions of isothiocyanates (ITCs) in the presence of amino compounds leading to coloured structures *via* substituted 2-thiohydantoins. A series of complementary experiments has been done and appropriate reaction conditions and structural prerequisites have been defined. Low-molecular colourants isolated and characterised from the model systems can be sorted into three groups. Yellow to red diastereomeric dehydrodimers of 2-thiohydantoin derivatives that contain an acidic methylene group are formed in mixtures consisted of ITCs and amino acids with α-methylene group in mild acidic to mild alkaline systems. The condensation products of the 2-thiohydantoins with reactive aromatic or heterocyclic carbaldehydes from the Maillard reaction, essential oils etc. comprise a heterogeneous group of mostly yellow colourants. Blue compounds of two types are structurally more complicated structures that arise from *N*-substituted amino acids and ITCs in alkaline media.

Keywords: isothiocyanates; amino acids; 2-thiohydantoins; carbaldehydes; colour; pigments

INTRODUCTION

Isothiocyanates (ITCs), physiologically active decomposition products of glucosinolates, are responsible for the pungent and acrid flavour of many vegetables and condiments of the *Brassicaceae* family (Velíšek 1995). ITCs are believed to possess a variety of biological effects (Duncan 1991). Recent data demonstrate a clear suppressive effect of many ITCs on mammary tumor models (Zhang & Talalay 1994). Thus, ITCs can be considered also as a class of phytochemicals offering significant hope in preventing the development of cancer. Owing to the electrophilic character of the ITC functional group, the toxicity and, on the other hand, the protective effect is assumed to be associated mainly with *in vivo* reactions of ITCs with thiols, amines and alcohols. The reactions of ITCs with these nucleophiles in foods during processing and storage are very frequent as well, and may result in reduction of nutritional value, formation of compounds with mostly unknown biological properties and changes in flavour as well as colour. ITCs react effectively with free amino acids, N-terminal amino acids bound in peptide chains, disulfide bonds etc. (Drobnica *et al.* 1977). The reactions can proceed during storage, cooking or food processing as well as in the human digestive system. The kinetics and mechanism of the formation of the *N*-substituted thiocarbamoyl amino acids and their cyclic forms, 3,5-disubstituted 2-thiohydantoins, were thoroughly described by Drobnica *et al.* (1977) and Cejpek *et al.* (2000a, b).

Despite recent considerable progress in the knowledge on discolouration in processed and stored foods (Adams & Brown 2007), more information focused on the structure and properties of discolouring pigments, especially in processed materials of vegetable origin, is needed. Within the vegetables of the *Brassicaceae* family, several sources of colour formation in selected *Brassica* and *Raphanus* species have been recognised so far. L-Ascorbic acid was identified as a key precursor of pinking and reddening of Brassicaceous vegetables *via* scorbamic acid (Kurata *et al*. 1973). Isothiocyanates are considered exceptionally in these pathways. Formation of yellow pigment containing a β-carboline skeleton was observed after reaction of 4-methylthio-3-butenyl isothiocyanate

with L-ascorbic acid and a dihydroxyphenolic compound (Ozawa *et al.* 1990).

MATERIAL AND METHODS

Reactants. Allyl isothiocyanate (allyl-ITC), butyl-ITC, phenyl-ITC (Fluka, CH), glycine, alanine, sarcosine (Merck, D), *N*-ethylglycine, *N*-acetylglycine, *N,N*-dimethylglycine (Lachema, CZ), furan-2-carbaldehyde and benzaldehyde (Fluka, CH) were used as reactants.

Model experiments. Aqueous models were binary solutions with 5–50mM concentrations of isothiocyanate and aminocompoud in buffered aqueous solutions of pH 4, 6, 8, 9, 10, 11 and 12, and stored at 4, 20 and 37°C. The systems of a 2-thiohydantoin and a carbaldehyde in 0.05M buffered aqueous solutions of pH 2–12 were stored at 20°C.

Analyses. The purification of reaction mixtures was performed on C_{18} Extract-Clean™ columns (Alltech, USA) and the obtained methanolic concentrates were analysed using HPLC/PDA and HPLC/MS systems. The red and yellow products were collected after separation of the concentrated extract by means of semi-preparative chromatography (Lichrospher 100 C_{18} in LichroCart 250-10, Merck). Fractions with blue products were purified using procedure based on the extraction by Et_2O prior to HPLC. XBridge™ C_{18}, 150 × 3 mm, 3.5 µm, and pre-column (Waters, USA) with gradient elution (10mM ammonia formate/acetonitrile), f = 0.4 ml/min, 20 µl loop, and HPLC system consisting of Waters 2 × 515 HPLC pump and 996 PDA detector system were used. The MS detector was Q-TOF Micro™ (Micromass, USA). Structure and purity of the isolated compounds were confirmed by ^{1}H- and ^{13}C-NMR spectrometry (Bruker Avance DRX 500).

RESULTS AND DISCUSSION

The main objective of this work was to characterise compounds responsible for the colour formed in the reaction mixtures. The study is also concerned with the definition of optimum reaction conditions and structural preconditions of the reactants, which are needed for the formation of these coloured secondary products in ITC-amino acid systems. Under alkaline and neutral conditions, 2-thiohydantoins (2-THs) converted partly to their open forms, thiocarbamoyl amino acids, and partly were transformed to other products. Among them, dimers of 2-TH coupled with single (non-coloured) or double (coloured substances) bonds through C-5 were revealed by structural analysis (Cejpek *et al.* 2000b). Non-coloured transformation products development of 3-allyl-5-methyl-2-thiohydantoin, which is formed from allyl isothiocyanate and alanine, was observed in neutral and alkaline solutions. The advanced products were identified as diastereomers of 1,1'-dimethyl-3,3'-dially-2,2'-dithioxo-[5,5']-biimidazolidine-4,4'-dione arising by oxidative dimerization of the 2-THs. If 5-unsubstituted 2-THs formed from amino acids containing C_α methylene group such as optically inactive glycine are oxidised, symmetrical coloured dimers are formed due to double bond formation between C-5 methylene atoms on the heterocycles. The changeover of yellow colour of the dimers in acidic media to the red colour at higher pH values is caused by thioxo-enol transitions (Cejpek *et al.* 2000a). We also tested the ability of other amino acids with C_α methylene group to give coloured products in the presence of isothiocyanates. We have revealed that both sarcosine and *N*-ethylglycine can yield red coloured products when reacted with ITCs in neutral and alkaline solutions. We identified one major (> 90% of absorbance at 500 nm) derivative of 1,1'-dialkyl-3,3'-dialk(en)yl-2,2'-dithioxo-[5,5']-biimidazolidinylidene-4,4'-dione in each of the system tested. The highest yields of the colourants were in allyl-ITC/sarcosine (m. w. 338 Da, λ_max_(pH_8)_487_nm), allyl-ITC/*N*-ethylglycine (m. w. 366 Da, λ_max_(pH_8)_498_nm), and butyl-ITC/sarcosine (m.w. 370 Da, λ_max_(pH_8)_487_nm) mixtures. The colour development in phenyl-ITC mixtures was much slower in comparison to the tested alk(en)yl-ITCs.

While *N*-acetylglycine and *N,N*-dimethylglycine are not able to form 2-THs and therefore any colour in ITC systems, sarcosine and *N*-ethylglycine are also precursors of blue pigments arising in alkaline solutions. The highest yield of the blue products with simultaneous minimum formation of interferences was obtained from the reaction mixture of 10mM allyl-ITC/5mM sarcosine in pH 11 after about 5 days at 20°C. Blue products of m. w. = 465 Da (mass isomers AS-Blue1, AS-Blue2 and minor AS-Blue4) and 446 Da (AS-Blue3) were formed in this mixture. Two kinds of blue colourants were recognised. Three blue products of allyl-ITC/*N*-ethylglycine mixture (AN-Blue1–3, 493 Da) are analogous to the former allyl-ITC/sarcosine products of m. w. 465 Da, while BS-Blue1 (494 Da) from butyl-ITC/sarcosine system corre-

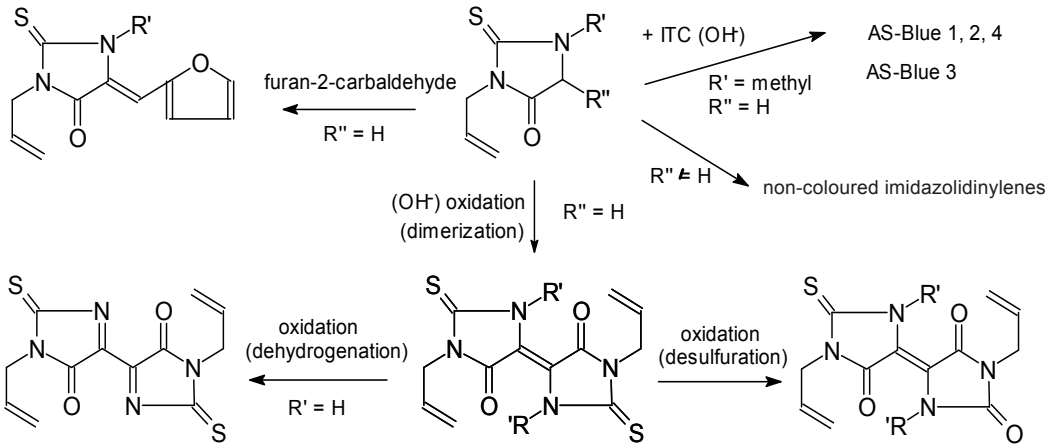

Figure 1. Transformation of 3-allyl-2-thiohydantoins to coloured structures

sponds to AS-Blue3. Spectral data, high-resolution MS methods and comparative analyses revealed that two molecules of the parent amino acid and three molecules of isothiocyanate are involved in the structure of the former kind of colourants. The latter is formed by three molecules of ITC and odd number of amino acid or its part. Despite high colour intensity, the yields were very low, therefore detailed structural analyses for identification of the blue products have not been yet allowed. Stability of blue colour depends on the structure of the colourants and the solvent used. Generally, they are more stable in alkaline (pH 11) than in neutral aqueous media. Concentration of AS-Blue1 and AS-Blue2 decreased to 12% and 13%, respectively, in pH 11 after 2 months' storage in a fridge, while levels of the colourants in water dropped to about 5%. Experiments with isolated AS-Blue compounds in solid state revealed higher stability when bound on inert supports.

C-5 non-substituted 2-THs also react willingly with aldehydes due to the base catalysed condensation on the active methylene group. Many 5-arylmethylene derivatives were prepared in this way (Avendano Lopez & Gonzalez 1985). The experiments accomplished in diluted aqueous solutions revealed satisfactory yields of condensation products of 2-THs with heterocyclic and aromatic aldehydes even in alkaline media, where fast cleavage of 2-THs and other transformations occur. For example, almost 25% of 3-allyl-2-TH react with furan-2-carbaldehyde in pH 10 within several hours. The 2-TH elimination is much slower (3 days) in pH 6, and about 75% is finally transformed to two yellow stereomers of 3-allyl-5-furfurylidene-2-thioxoimidazolidine-4-one (λ_max_392 and 398 nm, m. w. 234 Da). Analogous data were achieved for the systems containing benzaldehyde (Figure 1).

The knowledge of premises and conditions suitable for the formation of the colourants may be worthwhile in applications such as ITC-based amino acid determination and/or sequencing methods, and goal-directed production of alternative colourants from plant sources. However, due to low concentrations of coloured dimers at pH conditions common in foods, and competitive ITCs reactions in complex matrices, there are no great expectations on contribution of the mentioned coloured structures to significant discolouration of Brassicaceous foods at the conditions common for food processing and technology.

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