Bioactive sulfur compounds in broccoli and other *Brassicaceae* plants; the glucosinolates and S-methylcysteine sulfoxide as precursors of methylthiocyanate and dimethylsulfoxide

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Introduction

Cruciferous vegetables including broccoli and other plants of the family *Brassicaceae* accumulate appreciable amounts of structurally different bioactive sulfur compounds. Well known members of these groups of allelochemicals are the glucosinolates (Marks *et al*., 1992; Hansen *et al*., 1995; Bellostas *et al*., 2007; Andersson *et al*., 2008; and refs cited therein). Among the bioactive sulfur compounds are also some non-protein bound amino acids (Giovanelli *et al.*, 1980; Eggum and Sørensen, 1989; Hansen *et al.*, 2001). These groups of allelochemicals are precursors for characteristic smell, taste and other bioactive properties associated with the native compounds and products produced thereof, as known for the glucosinolates (Andersson *et al*., 2008b; Andersen *et al.*, 2010) producing isothiocyanates and sulfur containing amino acids producing garlic oils with specific taste and smell, e.g. as known for homogenized vegetative parts of Garlic mustard in cruciferous plants (*Alliaria officinalis* Andrz.). We need, however, more specific information on the structure-properties of the actual bioactive compounds in complex matrix systems, the metabolic control involved and chemical reactions on molecular level, including effects from stereochemistry (Andersson *et al.*, 2008; Andersen *et al.*, 2010). Important is as well the effects on the plant materials or matrix systems from post harvest conditions (Hansen *et al.*, 1995; 1997; 2001) and processing conditions.

Glucosinolates are well known precursors of thiocyanates, nitrils, epithionitriles, amines and reactive isothiocyanates (ITC´s) (Eggum *et al*., 1989; Bellostas *et al*., 2007; 2008; 2009). The ITC´s may then react with nucleophiles, and the ITC´s of indol-3-ylmethylglucosinolates are in very fast reactions transformed into the thiocyanate ion and a complex group of indolyls (Bellostas *et al*., 2007). In florets of broccoli varieties, the glucosinolates are quantitatively dominated by indol-3-ylmethylglucosinolates, which are the precursor of the thiocyanate ion. Quantitatively dominating are as well the S-methylsulfinylglucosinolates; glucoiberin and glucoraphanin (Hansen et al., 1995 and 1997), the precursors of the reactive ITC´s, (R)-3-methylsulfinylpropylisothiocyanate and (R)-4-methylsulfinylbutylisothiocyanate /sulforaphane (Eggum *et al.*, 1989). It has also been found that broccoli accumulate methylthiocyanate and dimethylsulfoxide (DMSO) as quantitatively dominating compounds in the group of sensory active broccoli compounds (Hansen *et al*., 1997). In the present work, focus has been placed on the potential precursors of methylthiocyanate and DMSO. The DMSO precursor is considered to be L or (2R) S-methylcysteine sulfoxide (L-SMCSO), the quantitatively dominating non-protein sulfur amino acid in broccoli florets, and the thiocyanate ion seems to be the precursors of methylthiocyanate. Both precursors are assumed to be substrates for S-methylation in S-methyltransferase (EC 2.1.1.10/ EC 2.1.1.12) catalysed reactions (Attieh *et al.,* 2000a; 2000b; 2002; Nagatoshi and Nakamura 2009) with S-adenosylmethionine (SAM) and/or S-methylmethionine (Vit.U) as cofactors /methyl donors. The methylsylfinylanion part of DMSO is considered to be a products of S-alkylcysteine lyase (EC 4.4.1.6) catalysed degradation of L-SMCSO, which is supported by the observation of acetaldehyde and ethanol as accumulated broccoli metabolites of pyruvate from the CS-lyase reaction (Hansen *et al*., 2001). In injured cells, SMCSO, is transfered into S-methyl sulfenic acid, pyruvate and ammonia in a CS-lyase reaction by action of S-alkylcysteine lyase (EC 4.4.1.6) (Marks *et al*., 1992), and following rearrangement of S-methyl sulfenic acid into the methyl-sulfinyl anion.

In this study focus has thus been on the reactions leading to the formation of DMSO, CH_3-SCN and the possible involvement of glucosinolate products, non-protein bound amino acid and thiol-methyltransferase

Materials and methods

Amino acids have been isolated by standard procedures (Eggum and Sørensen, 1989). Individual amino acids were determined as the dinitroflourobenzen (DNFB) derivatives by use of MECC, and for isolated amino acids by the MECC- cholate buffer method mentioned below using 210 nm as detection wavelength (Sørensen *et al.*, 1999). Glucosinolates were isolated and the individual compound determined by use of standard procedures (Hill *et al.,* 2003; Sørensen *et al.*, 1999). The DTAB-MECC procedure for determination of the thiocyanate ion is described elsewhere (Bjergegaard *et al.*, 1995). MECC synchronous determination of substrate and products is described in details elsewhere (Bellostas *et al.*, 2006).The applied crude enzyme preparations were produced from fresh broccoli florets as aqueous solutions of the ammonium sulfate precipitate produced as described by Attieh *et al*. (2000a; 2000b; 2002) and Nagatoshi and Nakamura (2009).

Results and discussion

In order to study the possible transformation reactions of DMSO and CH_3-SCN broccoli heads have been analysed for their content of pre-coursers of the reaction, the sulfur containing non-protein bound amino acids. An MECC assay was developed in order to synchronous and simultaneous follow substrates and products in the enzymatic formations of DMSO and CH_3-SCN.

Broccoli heads were analysed for content of non-protein bound amino acids, these were isolated and identified by preparative paper chromatography, and by different capillary electrophorsis methods. A MECC cholate system (Bellostas *et al.*, 2006) was used to separate the broccoli sulfoxides from other amino acids (Figure 1). This revealed that ((2R,ωS)-SMCSO isomer (I) co-occur with trace amount of the (2R,ωR) SMCSO (II). Spiking with standard compounds revealed that Vit. U has faster migration times (MT) than the solvent peak, and the S-adenosylmethionine (SAM) has MT slightly faster than the solvent peak, SMCSO has MT slower than the solvent front and was followed by a quantitatively minor compound.

The amino acids from broccoli crude extracts were further isolated by use of hyphenated column chromatography followed by dinitrobenzene (DNFB)-derivatisation and analysis be MECC (Sørensen *et al.,* 1999). The hyphenated column chromatography used for group separation consisted of a weakly acidic cation exchanger that captures the cations and basic amino acids, and then a strongly acidic cation exchanger to capture the neutral and acid amino acids. The MECC analysis of DNFB derived neutral and acidic amino acids is shown in figure 2. L-SMCSO migrated in front of the other amino acids, and was found to be one of the quantitatively dominating non-protein amino acids.

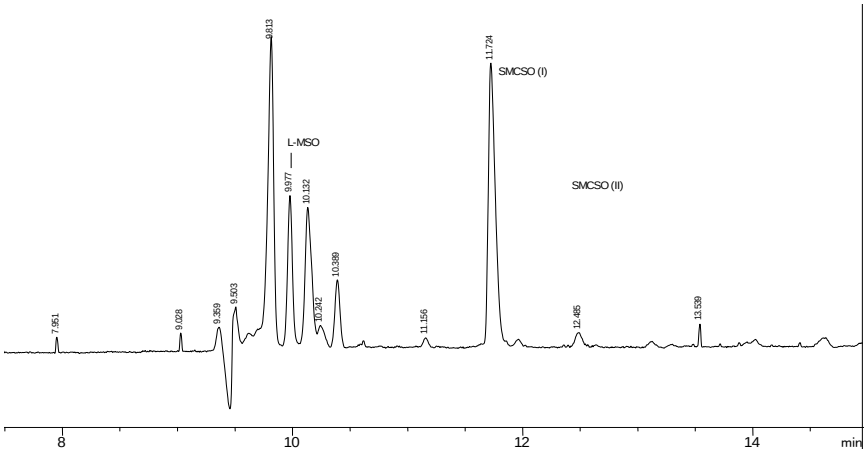

Figure 1. MECC-cholate system detection at 214 nm, used to separate the broccoli sulfoxides from other sulfur containing amino acids which occur in florets of broccoli

The MECC system (Figures 1) has also been used to investigations of the non-enzymatic reactions and potential enzyme catalysed formation of DMSO and methylthiocyanate. The assay systems have been based on the principles behind the MECC-synchroneous monitoring of substrates and products (Bellostas *et al.*, 2006).The formation of methylthiocyanate is assumed to be a product of S-methylation of the thiocyanate ion produced as a product from degradation of indol-3-ylmethylglucosinolates. The enzyme system considered for the S-methylation is the thiol methyltransferase (Attieh *et al.,* 2000a; 2000b; Nayatashi and Nakamuse, 2009).

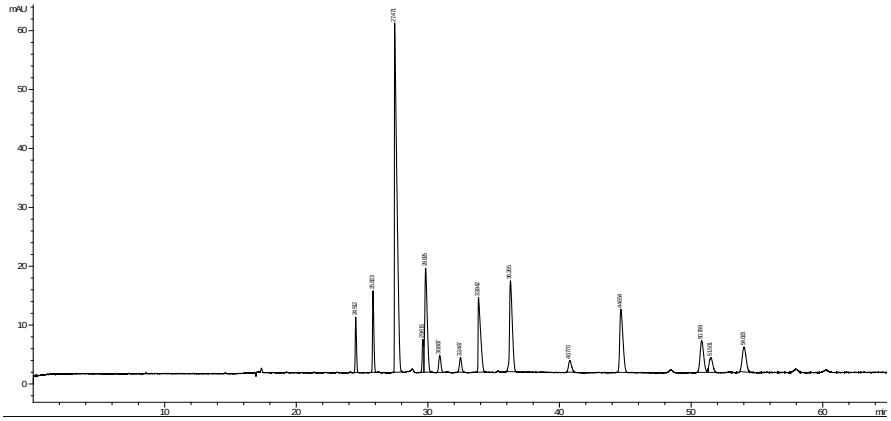

Figure 2. MECC of dinitrobenzene derivatives of non-protein bound broccoli amino acids. Norvaline was used a internal standard and L-SMCSO are migrating in front of the other amino acids.

The assay system was the ammonium sulfate precipitate produced as described in these papers, and with use of SAM as additional added cofactors, and potassium thiocyanate as substrate. The possibility of the reaction to occur as non-enzymatic methylation of the thiocyanate ion by SAM was tested both at room temperature and at 60 °C. With respect to the production of DMSO, a corresponding assay with broccoli ammonium sulfate precipitate, as CS-lyase source, PLP as cofactor and the SMCSO as substrate have been tested.

Methylthiocyanate (and potassiumiodid) synthesis as non enzymatic catalysed reactions were tested chemically at 60 °C by adding methyliodide to potasiumthiocyanate. These compounds were additionally analysed by MECC with a DTAB system (Bjergegaard *et al.,* 1995), for comparison with the assay described by Attieh *et al.*, 2000a. These authors analysed thiol-methyltransferase activity of KI with SAM as methyldonor by measuring the formation of CH_3I by GC. In the applied DTAB system CH_3I, KI and were well separated, and it seems to be possible to follow the enzymatic degradation of substrates as well as formation of products by the developed methods. However, additional trials are required for proper determinations of the potential enzyme catalysed production of DMSO and methylthiocyanate

Conclusion

Methods of analyses have been developed for measurement of thiol-methyltransferase activity in Brassica plants. Dimethylsulfoxide (DMSO) and methylthiocyanate (CH_3SCN) are produced in vegetative parts of Brassica plants; broccoli and rapeseed. The precursor of these compounds seems to be S-methylcystein sulfoxide for the production of methylsulfinylanion, as part of the CS-lyase reaction, and then production of DMSO in a thio-methyltransferase reaction. The latter enzyme reaction seems also to be the basis for production of CH3SCN from thiocyanate ion produced from indol-3-ylmethylglucosinolates.

Acknowledgements

The Commission of the European Union (FP6-NovelQ 015710-2), the Danish Agency for Science, Technology and Innovation (BioRef), the project Rapeseed Protein for Piglets, and Danish pig production, Axelborg, Denmark are gratefully acknowledged for the financial support of this work.

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