Use of Crucifers containing the glucosinolate-myrosinase system as a source of bioactive molecules

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Abstract

Over the last decade, the demand for sustainable, nonpolluting agricultural techniques able to provide healthful and safe products for the end user has notably increased. These features are in line with the new international regulations that require a progressive reduction of synthetic chemicals use in agriculture and in food technology. In addition, many natural molecules, including those obtained by biotransformation, were found mild, safe and reliable remedies to prevent or to fight a number of human diseases. In this context, the use of less dangerous procedures and chemicals are expected to become more and more important in the near future.

The beneficial effects of Brassicaceae use appears to be tightly associated with the content of myrosinase (MYR)-glucosinolate (GLs) system common in this plant family. This enzymatic system is important not only because it is one of the most significant protection structure in the plant kingdom, but also because it provides chemoprotection when assimilated with diet or useful outcomes in crop protection, particularly in biofumigation.

Biofumigation using cruciferous-derived materials appears to be a sound alternative technology for improving human health by reducing environmental problems. As a matter of fact, there has recently been an increasing interest for natural compounds with high biocidal activities as possible substitute for methyl bromide and other synthetic fungicides.

Although GLs are contained in all tissues of Brassicaceae, the seed is the preferential accumulation site. Environmental factors, plant species and age affect the GLs content and profile in Brassica seeds and tissues. Usually, intact GLs show low biological activity and chemical reactivity, while they are precursors of a number of bioactive molecules, and can break down by chemical, physical or enzymatic agents. All these reactions give rise to a number of different products depending on the type of degradation and reaction conditions, including isothiocyanates (ITCs). The latter are efficiently produced by the myrosinase-catalyzed hydrolysis of GLs and are valuable bioactive molecules that can be indirectly used in fine chemistry, food technology and crop protection.

At present, the potential of GLs is mostly exploited using Brassica tissues (sprouts, flowering plants, dry plant tissues and seed meals). However, suitable formulates with higher biological activity that would allow a wider number of agricultural applications, are possible using pure components or concentrated extracts of the GLs-MYR system. GLs extracts can be economically produced should the current seed crushing and extraction plants be slightly modified to accommodate the biorefining technology that enhances their fractionation.

Key words: Brassicaceae, Brassica oilmeal, Glucosinolates, Isothiocyanates, Biofumigation, Cancer protection

Introduction

The need for a sustainable agriculture that is eco-friendly and able to supply healthful and safe products has notably increased over the last decade. On the other hand, the new international regulations require a progressive and significant reduction of synthetic chemicals use in agriculture and in food technology.

Several recent papers indicate that many natural molecules, including those obtained by biotransformation, are effective not only in controlling and fighting a number of plant pathogens but also in preventing some important human diseases. The awareness of the benefits deriving from the use of less dangerous procedures and biobased biochemicals from agricultural renewable resources is expected to become more and more important in the near future.

In the middle of the nineties this approach was somewhat common in western countries where it efficiently helped to safeguard the environment without compromising agricultural economics. The next future agro-industrial benefits that can be expected from this innovation in agro-industry will depend mostly on the type and impact of improvement that will be possible to perform on plants and on process technology.

Indeed, some new and alternative extraction technologies show a great potential if applied to new plant genotypes improved for special uses, and to special co- and by-products that could be rediscovered and valorized for producing a number of high value molecules and materials. In this way, traditional and additional high value *green* products would be available; in the first case: oil, protein, starch, cellulose and their derivatives; in the second instance: dietetic fibers, special sugars, acids, and a number of bioactive secondary metabolites. Many of these products can be obtained from cruciferous oilseeds like *B. napus*, *B. carinata, B. juncea, Crambe abyssinica, Eruca sativa*.

The beneficial effects of the use of plants belonging to the Brassicaceae family in pharmacology, food technology and agriculture depends mostly on the myrosinase (MYR)--glucosinolate (GLs) system contained in their tissues. This enzymatic system is important not only because it is one of the most significant *in planta* protection structure in the plant kingdom, but also because it provides chemoprotection when assimilated with diet, in addition to being efficient when used in crop protection, particularly in biofumigation (Chew, 1988; Louda and Mole, 1991; Manici et al., 1997; Nastruzzi et al., 1996; Rosa et al., 1997; Barillari et al., 2005; Fahey, 2006; Palmieri, 2006).

Glucosinolates

There are over 120 known GLs all of which being S-glucopyranosyl thioesters secondary metabolites, which display a remarkable structural homogeneity: a common hydrophilic -D-glucopyrano framework bearing a O-sulfated anomeric *(Z)*-thiohydroximate moiety connected to a hydrophobic aglycon side chain that determines the structural molecular variation of GLs as aliphatic, aryl-aliphatic or heterocyclic atom arrangements (Tookey et al., 1980; Fahey et al., 2001; Rollin and Palmieri, 2004).

In their native form, GLs have a low biological activity, while their degradation products: isothiocyanates, thiocyanates, nitriles and epithionitriles, obtained by MYR-catalyzed hydrolysis, represent an important group of bioactive molecules of vegetable origin (Figure 1). So far, GLs enzymatic degradation products have been studied mainly for their antinutritional effects in animal feed, although in recent years these compounds proved to be valuable due to their interesting biological and chemical properties. A number of authors consider these molecules very useful, not only for their activity against bacteria, fungi, nematodes, and their potential in cancer prevention (Fenwick et al., 1983; Lazzeri et al., 1993; Huang et al., 1994; Leoni et al., 1997, Barillari et al., 2005), but also because some of them can be used as important intermediates in chemical synthesis (Gueyrard et al., 2000).

The mechanism of GLs enzymatic hydrolysis has been studied using the main MYR isoenzyme isolated from white mustard (*Sinapis alba*) (Palmieri et al., 1986; Pessina et al., 1990) and crambe (*Crambe abyssinica*) (Bernardi et al., 2003) seeds and various types of GLs, desulfo-GLs and some synthetic competitive inhibitors (Iori et al., 1996; Cottaz et al., 1996).

The molecules produced in these MYR-catalyzed reactions have interesting pharmacological properties, which make them potentially useful for different innovative applications. The positive and negative effects associated with the GLs content and composition, as found in food, feed, and vegetables brought to focus the efforts to clarify the relationship between chemical structure and biological activity (Sørensen H. 1990; Bjergegaard and Sørensen, 1996).

Myrosinase

MYR (Thioglucoside glucohydrolase E.C. 3.2.1.147) catalyzes the cleavage of the thioglucosidic bond (-S-Glucose) from GLs via acid/base catalyzed reaction with the release of the aglycon and the formation of the glycosyl enzyme intermediate (Burmeister et al., 2000). MYRs, together with GLs, are present in all species of the *Brassicaceae* plant family and are particularly abundant in the seeds where they are synthesized in the endoplasmatic reticulum. Enzyme and substrates are normally stored in different cell sites, and when they are mixed following a mechanical wounding or pathogen attack of plant tissues, they generate cytotoxic breakdown products that play an important role in plant defense system (Bones and Rossiter, 1996). MYRs are stable glycopolypeptides containing various disulfide and salt bridges, as well as a high percentage of carbohydrates, mainly hexoses. In *Sinapis alba* seeds, MYR is a dimer in solution with two identical subunits of 71.7 kDa and 499 residues, each stabilized by a Zn^{2+}ion bound on a 2-fold axis with a tetrahedral co-ordination (Björkman, 1972; Palmieri et al., 1986; Pessina et al., 1990; Burmeister et al., 1997).

Sinapis alba and other cruciferous seeds are good sources to isolate MYR in high purity and yield. Due its special physicochemical properties, MYR can be usefully used almost as an industrial catalyst. It may be used simply free in water solution, solubilized in reverse micelles, or in immobilized forms on organic and inorganic supports, depending on physicochemical characteristics of starting GLs, and mainly on the final GLs breakdown products, especially their water solubility and inhibitory properties on MYR.

MYR shows extraordinary standing and operational stability, which strongly indicates the high potential of this enzyme in large-scale processes (Leoni et al., 2000). With a potential huge availability of different kinds of GLs as industrial co-products, the GLs-MYR system appears to be an efficient and bio-friendly way to produce a wide assortment of added-value natural bioactive products, suitable for pharmaceutical and agro-industrial purposes, with a potential benefit to human health and quality of life (Palmieri et al., 1998).

The myrosinase-glucosinolate system

The MYR-GLs system is present in native form in brassica plant fresh tissues that can be directly used in green manure and biofumigation (Lazzeri et al., 2004; Kirkegaard and Matthienssen, 2004; Palmieri, 2006). The components of this enzymatic system after their isolation can be formulated with suitable techniques and used not only in crop protection, but also in fine chemistry and food technology. In plant cells, MYR and GLs are located in distinct cell sites, ready to interact in case of cell damage. Luthy & Matile in 1984 described the organization of this system as a microscopic “bomb”, which plays an important protection role in Brassica plant against many pathogens and phytophagous insects.

A suitable assembly of MYR-GLs system in liposomes, microspheres, or multiple emulsions appears to be a good tool to get a viable, cost-effective GLs-MYR formulation, that can be used in crop protection, with the following advantages: i) reduction of mammals toxicity; ii) decrease of phytotoxicity; iii) extension of activity duration; iv) cut of losses due to evaporation. Nevertheless to produce these formulations in large amounts, it is fundamental to have an efficient and economic procedure to isolate GLs and MYR at industrial scale. The possibility to reduce their extraction cost is realistic only if a number of other oilseed components such as oil and protein are extracted together with the most valuable compounds.

Benefit in human nutrition and anticancer activity

Although important improvements in cancer therapy have been realized in these last decades, prevention appears to be yet a necessary strategy to mitigate this affliction. According to the *American Cancer Society* guideline, one approach to cancer prevention is the diet optimization giving priority to plant-based food. In particular, a diet rich in cruciferous vegetables such as Brussels sprouts, cabbage, broccoli, turnip and cauliflower may have important functions in reducing the risk of developing not only colorectal cancer, but also some other degenerative diseases in humans.

The beneficial effects of these vegetables appear to be strictly correlated to GLs content and especially to some GLs degradation products, which are formed *in vivo* through GLs metabolization catalyzed by endogenous MYR enzymes or by those enzymes with MYR-like activity that are contained in commensal microflora. GL-derived isothiocyanates are important for they activity in cancer prevention because they are able to enhance the activity of phase II enzymes, which are involved in the detoxification of xenobiotic compounds assimilated with diet (Brooks et al., 2001; Bacon et al., 2003).

The potential of GLs and their degradation products in cancer therapy was also observed in our earlier studies carried out on proliferating human erythroleukemic K562 and other tumoral cell lines, using eight GLs degradation products obtained from MYR-catalyzed hydrolysis of pure GLs applying the *pre-mix* and *in situ* techniques. The enzymatic GLs breakdown compounds demonstrated a clear inhibition of proliferative cell growth. This inhibition was particularly evident for degradation products obtained from sinigrin, glucotropaeolin, glucoerucin, glucocheirolin, (IC_{50} < 20 μM) (Nastruzzi et al., 1996; Leoni et al., 1997).

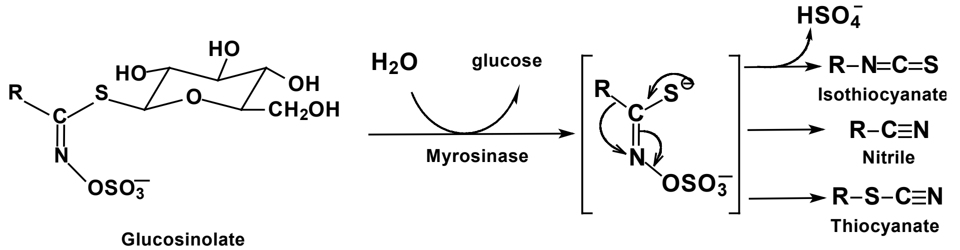

Figure 1 - General scheme of myrosinase-catalyzed hydrolysis of glucosinolates.

Soil biofumigation and food technology

The interest for natural compounds with high biocidal activities as possible substitute for methyl bromide and other synthetic fungicides, has come of age. From this point of view, cruciferous-derived materials appear to be a sound alternative resource for improving human health while contributing to the reduction of environmental pollution.

Biofumigation is a technique based on the use of cruciferous-derived materials containing cytotoxic plant metabolites such as GLs and their degradation products, mainly isothiocyanates (ITC), obtained by the action of MYR. Cruciferous species can be exploited in agriculture as green manure, rotation crops and as an alternative to the fumigant methyl bromide (MeBr) for controlling soil-borne pathogens (Kirkegaard et al., 1993; 1998; 1999; Manici et al., 1998; Martin 2003). Biofumigation has received an increasing interest also for other applications in agriculture, like in weeds and noxious insects control, ever since the use of MeBr has been forbidden in 2005 for western countries.

Recently, biofumigation has been tried also in food technology, the rationale being that some molecules deriving from the action of MYR--GLs system would control a number of food pathogens (Mari et al., 1993; 1996; Delaquis and Mazza, 1995). In particular, this technology was studied to control the post-harvest fruit pathogens using plant material or plant extracts containing the MYR--GLs system (Mari et al., 2002; Bernardi et al., 2004; 2005). The allyl ITC, that is formed by MYR-catalyzed hydrolysis of sinigrin, was the most effective in post-harvest fruits protection. On the basis of this result, the performance of a pre-pilot plant suitable for applying the post-harvest biofumigation technology was determined. With this tool, it has been possible to set up efficient procedures to control *Monilinia laxa* and *Penicillium expansum* on stone fruits and pears, respectively (Mari et al., 2006).

Extraction Technology

At present, the beneficial effects of GLs are mostly exploited in crop protection using Brassica tissues, sprouts, flowering plants, dry plant tissues and seed meals in green manure and biofumigation. However, suitable formulates characterized by a higher biological activity are near at hand, especially if a suitable and cost effective extraction technology is set up. This means that a wider number of agricultural applications are possible using pure components or concentrated extracts containing the GLs--MYR system in appropriate formulations. GLs/MYR extracts can be economically produced particularly if current seed crushing and extraction plants are properly modified so as to work applying the biorefining technology based on enzymes mixtures in aqueous solutions or reverse micelles dissolved in hydrocarbon solvent (Palmieri et al., 2003; Palmieri and Ugolini, 2006). In this way, the fractionation potential of extraction plants will notably increase which would reduce the cost of the extracts.

Concluding remarks

GLs are precursors of molecules endowed of a wide and strong bioactivity spectrum, which is dependent on the chemical structure of their functional group. A number of experiments carried out using pure GLs and their degradation products, demonstrate that the biological activity of these compounds can be advantageously exploited not only in crop protection as a possible alternative to MeBr, but also in fine chemistry for pharmaceutical applications, and in food technology. In particular, thiofunctionalised GLs (Glucoiberin, Glucocheirolin, Glucoerucin, Glucoraphenin), some alchenyl GLs (Sinigrin, Glucocapparin), and benzyl GLs (Glucotropaeolin, Gluconasturtin) are precursor of GLs degradation products that show the highest biological activity, and can therefore be considered as the most important candidates to be used as speciality chemicals with an important economic value. Some studies are in progress in our laboratory for explaining the mechanism of action of these molecules *in vivo*, although it is already known that one of the most important properties of these molecules lies in their increased hydrophobicity that makes it easier for them to penetrate through the hydrophobic lipid bilayer membranes.

At present, the easier way to employ the GLs-MYR system is to use plants containing this enzymatic system in green manures. This soft method to fight plant and soil pathogens (biofumigation), appears to be practical due to its good agronomic, economic and environmental attributes. Other more complex products containing the MYR-GLs system could doubtless, be forthcoming.

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