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Enzymes and Rapeseed : Reality and Potential

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Summary

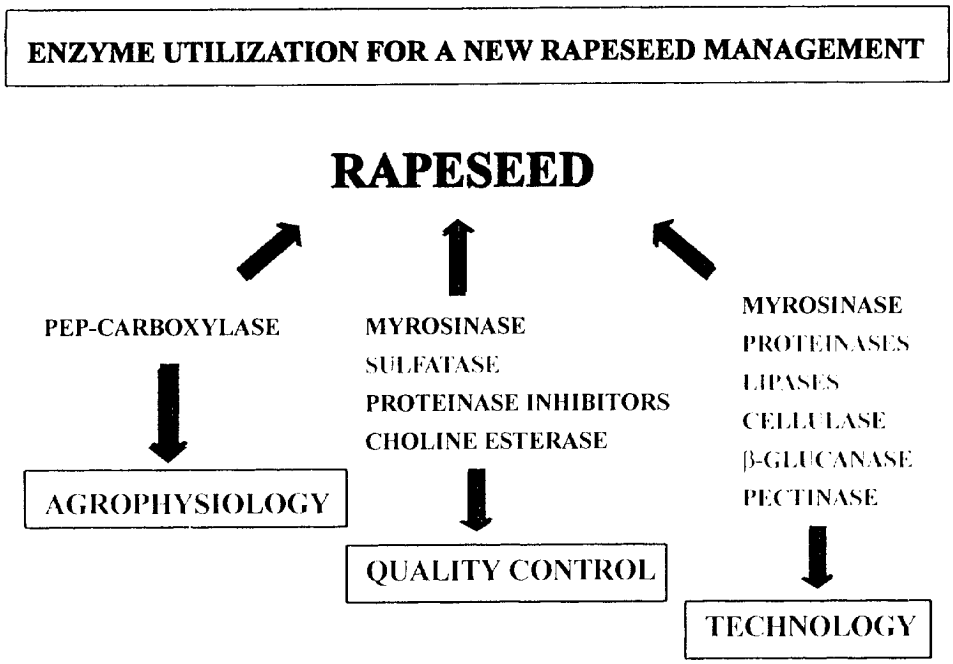
In recent years, enzymes are currently used in fine and agro-chemical industries. In the case of oil seed rape, there are at least three enzymes that can be useful to study the relationship genotype-environment, to improve the accuracy and reproducibility of glucosinolate (GL) HPLC analysis and to extend the range of products which can be obtained from oil seed rape processing. These enzymes are phosphoenolpyruvate carboxylase (EC 4.1.1.31) (PEPC), sulfatase (EC 3.1.6.1) (SUL) and myrosinase (EC 3.2.3.1) (MYR). Previous studies demonstrate that PEPC, although a typical enzyme of C4 and CAM plants, is also present in oil rape seed during seed development, where it plays a crucial role in the carbon balance, affecting final seed yield. As regard the analytical aspect, it has been demonstrated that purified SUL from *Helix pomatia* can be efficiently immobilized on Nylon 6.6 and used in this form to standardize the HPLC method for GL analysis. Finally, SUL and MYR, in soluble or immobilized form, seem to be an efficient system for producing interesting bio-active intermediates for further chemical modifications.

Introduction

Enzymes are an important subject in several disciplines such as biochemistry, molecular biology, microbiology and chemical engineering. Today enzymes are advantageously used in fine chemical industries, food technology, biochemical analyses and in plant biochemical genetics as molecular markers.

In oil seed rape, there are a number of enzymes, and their effectors, used in agrophysiology, to define seed quality, and improve the technology applied to this important oil-bearing seed crop. These active proteins can be endogenous as PEP-carboxylase, myrosinase, choline esterase and proteinase inhibitors or exogenous such as sulfatase, cellulase, β-glucanase and pectinase. Proteinases and lipases can be of both types (Fig. 1).

Fig. 1 -



Agrophysiology

Phosphoenolpyruvate carboxylase (PEPC) appears to be an important enzyme in the agrophysiology of this oleaginous plant.

Although PEPC is a typical enzyme of C4 and CAM plants, our previous studies demonstrate that it is also present in oil seed rape during seed development, where it plays a crucial role in the carbon balance, affecting final seed yield (1,2). In fact, as in other plants, PEPC also catalyzes PEP carboxylation by recapturing part of the CO_2 respired, which has accumulated in the pod cavity and in the cytoplasm, as $^-HCO_3$, during seed ripening (3). Thereby, in principle, an extra production of carbon skeletons is also available for fatty acid synthesis. In addition, previous studies on other plants report that light and nitrate anion, positively affect PEPC activity (4). In rapeseed, we determined that the developing seeds in the secondary branches showed a significantly lower PEPC activity than those of the main branch, which in general receive more light and nitrate anions. This last aspect is due to the well known phenomenon of apical dominance. We also established that a suitable nitrogen dressing increased PEPC activity in ripening seeds of secondary branches, without producing any significant enzyme activity change in the developing seeds of the main branch.

These findings would indicate that a suitable nitrogen dressing, at the right time and in the right amount, can give real yield optimization.

Quality control

The nutritional value of rapeseed derived products is significantly affected by the presence of some endogenous active proteins, the most important are myrosinases (MYR), choline esterases and proteinase inhibitors. In addition, MYR and sulfatase (SUL) are important enzymes for quality control because they can be used to determine the glucosinolate (GL) content in seeds and meals (Fig. 2).

The presence of MYR in the seeds is a critical aspect because, if it is not promptly deactivated, it can have a strong negative effect on the quality of rapeseed oil and proteinic defatted meal. In fact, MYR efficiently catalyzes the hydrolysis of rapeseed GL, mostly producing vinyl oxazolidine-2-thione and isothiocyanates (Fig. 3). This is true, even if 00 varieties (which contain less than 20 μmoles/g of GL in defatted meal) are correctly extracted with the usual processes. Thus many authors report that some μmoles/g of GL-DPs are normally present in the defatted meal (5).

Fig. 2 - Enzyme used to define the quality of rapeseed and derived products

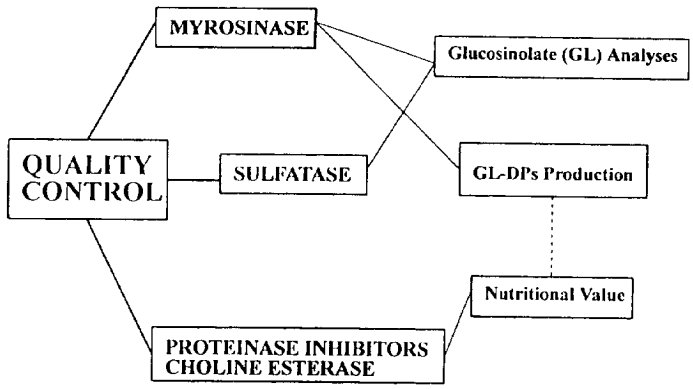
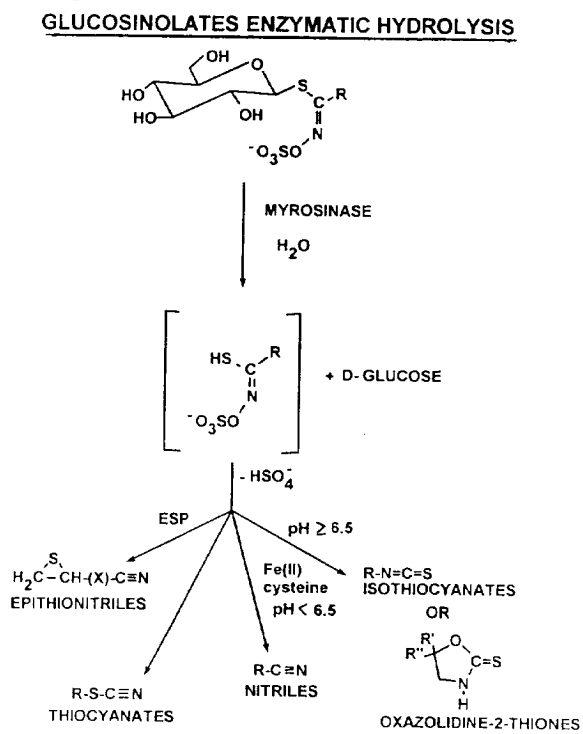


Fig. 3 -

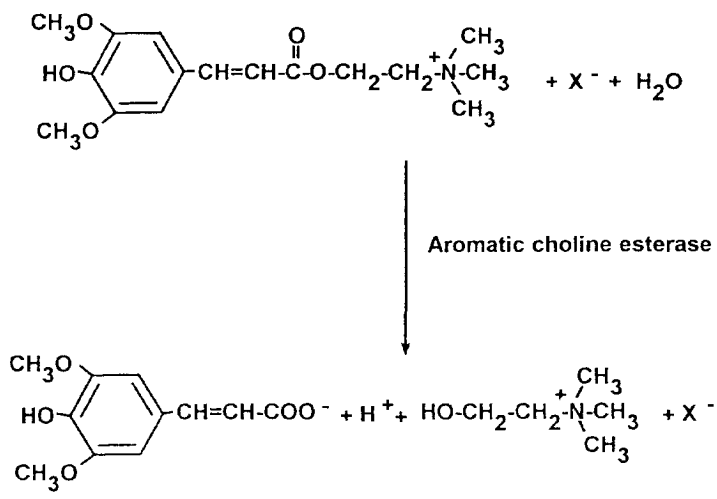


Recently, we isolated and characterized a new family of proteinase inhibitors from cruciferous seeds. Among these inhibitors, those contained in rapeseed are very interesting; mostly for nutritional implications (6). The average value of trypsin inhibitory activity (TIA) in six rapeseed genotypes was about 4.5 TIA. This activity was essentially due to three distinct isoinhibitors, which were easily separated by gel filtration FPLC (7). Nevertheless, some genotypes showed a TIA about double of the average value, which was roughly that reported for toasted soybean protein concentrates. However, from the nutritional point of view, the most important aspect is that the main inhibitor, which was responsible for 85% of the total activity, was completely thermostable. Clearly, this means that the digestibility of proteinic meals cannot be improved simply by thermal treatment.

The third active protein important for rapeseed quality is choline esterase because it is able to hydrolyze sinapine (Fig. 4). Sinapine is a choline ester of sinapic acid and it is a well known constituent of rapeseed since it is associated with the bitter taste of ground rapeseed. Sinapine is contained in rapeseed in a concentration of about 1% (8), and when it is hydrolyzed by choline esterase it gives a hydroxylated tetramethylamine, which has high negative organoleptic properties (like rotten fish). Fortunately, there seems to be little choline esterase activity in ripe seed, whereas this activity becomes much higher during germination (9). This means that seeds have to be correctly processed and stored before extraction to avoid a crabby and fishy odour of the proteinic meals.

Fig. 4 -

SINAPINE ENZYMATIC HYDROLYSIS



During the last decades, analytical methodology for GL determination was significantly improved by the use of MYR and SUL. MYR was used to set up a rapid method for the simultaneous analysis of total free glucose and total GL in aqueous extract of rapeseed (10). This technique involves precise and reliable polarographic determination of O_2 uptake, following free glucose oxidation by a system of double-coupled enzymes such as myrosinase--glucose oxidase.

As regards the determination of individual GL in cruciferous material, it has been demonstrated that HPLC analysis of desulfo-GL (DS-GL) has some advantages as compared with the same method applied to intact GL and, for this reason, it has been chosen as the reference method in the ambit of the EU (11). Thereby, for several years SUL has been used to produce DS-GL before HPLC analysis.

Recently, we demonstrated that purified SUL from *Helix pomatia* can be efficiently immobilized on Nylon 6.6 and used in this form to prepare standard desulfo-GL but, most important to improve the HPLC method for GL analysis (12, 13). The use of immobilized SUL, applied to the HPLC analysis of GL in cruciferous material, notably reduces analysis time. We established that 15 min are sufficient to remove the sulfate anion from all native GL in crude rapeseed extract. Otherwise, using the classical method, and in particular the reference method ISO 9167-1, this sample preparation step takes 15-18 hours, generally carried out overnight. This aspect is not marginal because sometimes, for various reasons, it is important to know the GL content of a sample of seed, meal or green vegetable quickly.

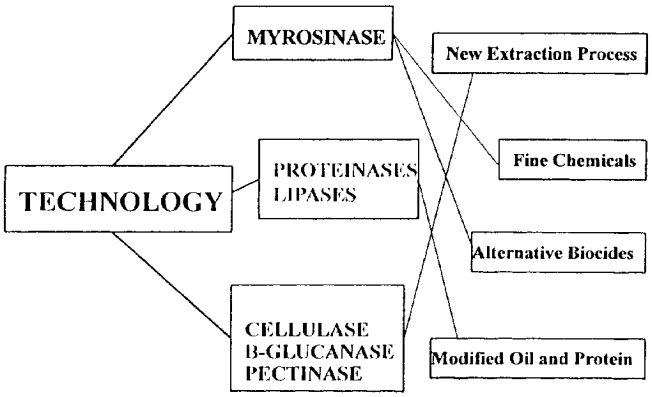
In addition, the method which uses immobilized SUL improves both GL recovery and analysis reproducibility. In fact, in samples tested with immobilized SUL, we recovered significantly higher GL contents with lower standard deviation values. The higher values obtained with the immobilized SUL method can be essentially attributed to the better recovery of indolyl-GL, viz. glucobrassicin, 4-hydroxy glucobrassicin and neo-glucobrassicin. The classical method, which uses free SUL, takes longer time and for this reason indolyl-GL are presumably degraded during the desulfation process.

Technology

In recent years a new concept for an optimal and environmentally safe utilization of cruciferous oil bearing seed, and of rapeseed in particular, has been developed. Some original technologies, based on enzymes utilization, have been studied and in some cases set up, for a pre-industrial production of new and high value products.

New extraction processes, based on the use of cell-wall degrading enzymes, have been applied to avoid the use of organic solvents in oil extraction and to improve the quality of the products. Interesting fine chemicals and alternative biocidal molecules could be obtained using MYR. Finally, modified oil and proteins are easily obtained using commercial proteinases and lipases (Fig. 5).

Fig. 5 - Enzymes useful in technology for producing improved or new rapeseed derived products



The most commonly used method for rapeseed processing involves the prepressing of flaked and cooked seeds, followed by solvent extraction of oil from the pressed cake. It is known that the energy required for this process is high and the quality of the extracted oil and meal is lower than that obtained with other methods. Meal quality is reduced by the high temperature required to remove hexane.

Cell-wall degrading enzymes were successfully used both as pre treatment to enhance oil extractability in conventional extraction processes and as the basic point of a new aqueous extractive technology for oil bearing seeds (14,15). In these applications, cell-wall degrading enzymes, essentially cellulases, β-glucanase and pectinase, favour the release of lipids, proteins and other water soluble molecules from plant material to aqueous suspension. All these enzymes are of microbial origin and are commercially available. They have an optimum activity at acidic pHs, in a range of 3.5 to 6.0.

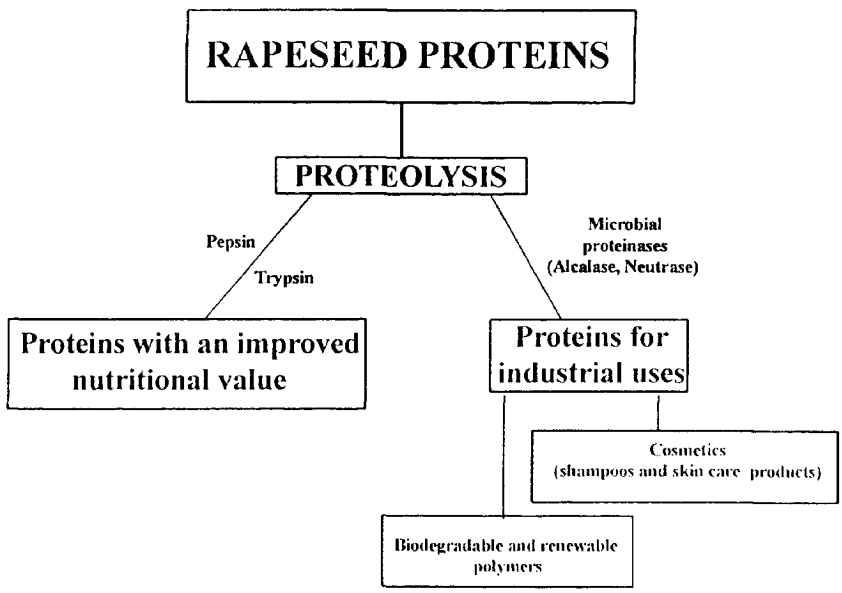
Technology involving cell-wall degrading enzymes for the aqueous extraction process appear to be the most innovative. In fact, this technique makes it possible to avoid the use of organic solvent and to produce several high quality rapeseed products, without negative effects on the environment. This means lower waste and energy consumption. The process provides a high quality oil and a dehulled protein-rich meal, practically free of GL and their degradation products, phenolics and other low MW compounds, including GL.

Proteolytic and lipolytic modification of food proteins and lipids is a very old technique, and often adopted and controlled by man, to improve taste and aroma of some important proteinic food products. The most important examples are fermented cheeses and processed meats, although other exotic foods, such as *shoyu* and *miso*, which are Japanese soy derivatives, are examples of proteolytically modified foods.

In principle, some functional properties of rapeseed proteins isolated from defatted meals could also be greatly changed and improved from several points of view, by modifying their physicochemical characteristics with proteolytic enzymes (Fig. 6). Although rapeseed proteinic meals are largely available, they are practically unknown in human nutrition, being essentially used as feed-grade goods. Nevertheless, some studies demonstrate that their nutritional value, experimented on rats, can be improved when hydrolyzed with pepsin and trypsin. The soluble low molecular weight protein hydrolyzate shows a significantly higher nutritional value than the original raw material, comparable with that obtained with casein (16).

Because at present the non-food use of plant proteins seems to be more and more important, the possibility of modifying these proteins by proteolytic enzymes to improve a particular functional property assumes great importance for the exploitation of this renewable resource. Although hydrolyzed plant proteins can be used in several industrial products, one of the most important uses is as a constituent of some cosmetic products. Important applications have been found in shampoos and skin care products (17).

Fig. 6 - Uses of modified rapeseed proteins after controlled proteolysis



Extra-cellular microbial lipases are currently used as catalysts, in free and immobilized form, for the enzymatic modification of several oils and fats. Lipases catalyze the hydrolysis of fatty acid esters, among these glycerides. Nevertheless, as for many other enzyme-catalyzed reactions, these reactions are reversible and, thereby, esterification and trans-esterification reactions can be also easily catalyzed by lipases.

Lipase-catalyzed reactions have been applied to trans-esterification processes for the production of high value triglycerides, used in the formulation of confectionery fats. In a typical process, a 1:1 mixture of high oleic vegetable oil and stearic acid it is possible to produce a cocoa butter substitute, which can be separated by crystallization to give a stearine containing a high concentration of the rare triglyceride StOlSt, one of the expensive components of cocoa butter. The same technique can be used to prepare triglyceride mixtures with useful functional properties for products such as special margarines, low calories and bakery fats.

Recent studies on the technological application of MYR demonstrate the possibility of using this enzyme, in the soluble and immobilized form, to efficiently hydrolyze GL (18), which could soon be available in large amounts. In fact, there is growing interest in some cruciferous industrial seed oils with high erucic acid content, as an alternative to other oils. In addition, the tendency to apply new seed processing technologies, which also make it possible to separate proteins from GL, strongly encourage these prospects.

All this considered, the use of the MYR-GL system appears to be an efficient and ecological way to obtain a large number of active naturally produced compounds (Fig. 3).

Relatively high amounts of MYR can be easily isolated from white mustard seeds (*Sinapis alba*) by affinity chromatography using ConA-Sepharose (19).

Our previous studies demonstrate that MYR has been successfully immobilized on several inorganic and organic materials and in this form can be used to produce a great number of GL derived products (DP) (20,21). Using one of these techniques, it is quite easy to produce a special GL-DP. It is important to choose the starting GL and the method. For instance, to prepare aliphatic isothiocyanates (ITC) or thiones, the use of MYR immobilized on Nylon appears to be the most advantageous, whereas MYR immobilized on gamma-alumina is suitable for nitrile (NI) preparation, since only be used in acidic pHs. Finally,

MYR dissolved in organic solvent, using reverse micelles, appears to be the best method for producing low water soluble ITC and NI (22).

Some GL-DP have been tested for their biological activity. They have been found active *in vitro* against sugar beet nematodes (*Heterodera schactii*), pathogenic fungi in the soil and in some fruits during the postharvest period (23,24,25,26). In addition, our recent studies demonstrate their activity in controlling the proliferation of some tumoral cell lines (27).

As regards rapeseed GL, which we find in general to have a medium low activity, interesting results were obtained in controlling *Aphanomicaes pisi* oospores with defatted rapeseed proteinic meals (28).

The technological application of the MYR-GL system is particularly interesting, when hydroxy-GL are used as starting material. As is shown in the scheme in Fig. 3, from progoitrin, the main GL of rapeseed, it is possible to obtain three or four different enantiomerically pure molecules. These compounds are of great interest because they are ideal to be modified for preparing molecules with improved bio-active properties (29).

Concluding remarks

In general there has always been a close association between the discovery of an enzyme with its catalytic mechanism and the development of agricultural biotechnology. From some decades, new and old enzymes have been used extensively in crop physiology, plant genetics and agro-technology. I have attempted to explain that this is also true for rapeseed, where the use of the aqueous-enzyme extraction process appears to be one of the most important application of enzymes. This new methodology seems to be now quite mature for application on an industrial scale. The use of this new technology, in addition to the greater environmental benefit, opens up other interesting possibilities as regards the use of rapeseed co-products.

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