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Inspired by Nature: The Use of Plant-derived Substrate/Enzyme Combinations to Generate Antimicrobial Activity *in situ*

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The last decade has witnessed a renewed interest in antimicrobial agents. Plants have received particular attention and frequently rely on the spontaneous enzymatic conversion of an inactive precursor to an active agent. Such two-component substrate/enzyme defence systems can be reconstituted *ex vivo*. Here, the alliin/alliinase system from garlic seems to be rather effective against *Saccharomyces cerevisiae*, whilst the glucosinolate/myrosinase system from mustard appears to be more active against certain bacteria. Studies with myrosinase also confirm that enzyme and substrate can be added sequentially. Ultimately, such binary systems hold considerable promise and may be employed in a medical or agricultural context.

Keywords: Alliin, Alliinase, Antimicrobial activity, Binary system, Glucosinolate, Myrosinase.

During the last decade, it has become apparent that most conventional antibiotics are not able to meet the demands of the coming years. A renewed interest in antimicrobial agents has stimulated extensive research, with a particular focus once more on plants and their biologically active secondary metabolites. Here, phytoanticipins have received particular attention. Such often highly toxic and aggressive compounds are not stored in the plant, but are generated on demand, mostly by enzymatic conversion of an inactive precursor to the active agent(s). There are several prominent examples of such two-component substrate/enzyme plant defence systems. The alliinase (EC 4.4.1.4) based defence system found in garlic and related *Allium* species, for instance, relies on the conversion of the mostly non-toxic sulfoxide alliin (*S*-allyl-L-cysteine sulfoxide) into the highly aggressive thiosulfinate allicin (2-propene-1-sulfinothioic acid *S*-2-propenyl ester) (Figure 1) [1,2]. As alliin and alliinase are stored separately in the intact garlic bulb, this defence only becomes active during an attack, when considerable local concentrations of allicin are produced within seconds to fight the attacker [3-5]. A similar defence system is found in mustard and rape seeds, where myrosinase (EC 3.2.1.147) converts various glucosinolates to highly aggressive isothiocyanates, thiocyanates, cyanates, indoles and related products (Figure 1) [6]. Whilst allicin and its chemical decomposition products interact preferably with redox sensitive cysteine proteins of the cellular thiolstat, the isothiocyanates and related electrophiles also target various amino groups (e.g. in lysine) [7]. In both cases, widespread posttranslational modifications of (sensitive) proteins and enzymes occur, aversively affecting the cell or organism and often resulting in (cell) death. Such toxicity of allicin, sulforaphane and their related products has been observed in many studies employing lower organisms, such as bacteria and fungi, but also in certain nematodes and insects [8-10]. In contrast, higher organisms, including humans, are able to tolerate allicin and the various isothiocyanates rather well as they contain cellular "buffers" against such chemical stresses, such as glutathione (GSH).

Ultimately, binary systems combine a number of aspects which may also enable an application in medicine or agriculture. Alliinase has already been studied extensively in this context, for instance against cancer cells and the rice blast fungus *Magnaphorte grisea* [11]. Yet reports of other substrate/enzyme combinations and applications to fungi and small organisms, such as nematodes, are seemingly rare. In order to investigate the efficiency of such binary systems against different types of micro-organisms, we have therefore compared the respective activities of the alliin/alliinase and glucosinolate/myrosinase systems against selected bacteria, yeast and the model nematode *Steinernema feltiae*. We have also posed the question if a combination of the two defence systems may result in a certain synergy, and if such a binary system could be developed into a more sophisticated, targeted system with substrate and enzyme being delivered separately.

Overall, our studies indicate that it is possible to isolate and partly purify both alliinase and myrosinase with considerable ease and to use these enzymes together with their respective synthetic substrates to kill microorganisms and nematodes rapidly and effectively, whilst neither enzymes nor substrates on their own show any significant activity. Both plant-derived systems seem to be fairly active, with substrate concentrations required for activity in the hundred micromolar concentration range. Still, besides similarities, there are also significant differences between them. Whereas a combination of alliinase with its substrates alliin or the propyl-analogue PCSO (*S*-propyl-L-cysteine sulfoxide) is particularly effective against *Saccharomyces cerevisiae*, it is rather ineffective against bacteria such as *Escherichia coli*. In contrast, myrosinase and its substrate(s) seem to be remarkably effective against the nematodes employed. Interestingly, it also appears that myrosinase, in particular, could be used to develop a more sophisticated two-component system since this enzyme can be applied well separated from its substrate. These findings will now be discussed in more detail.

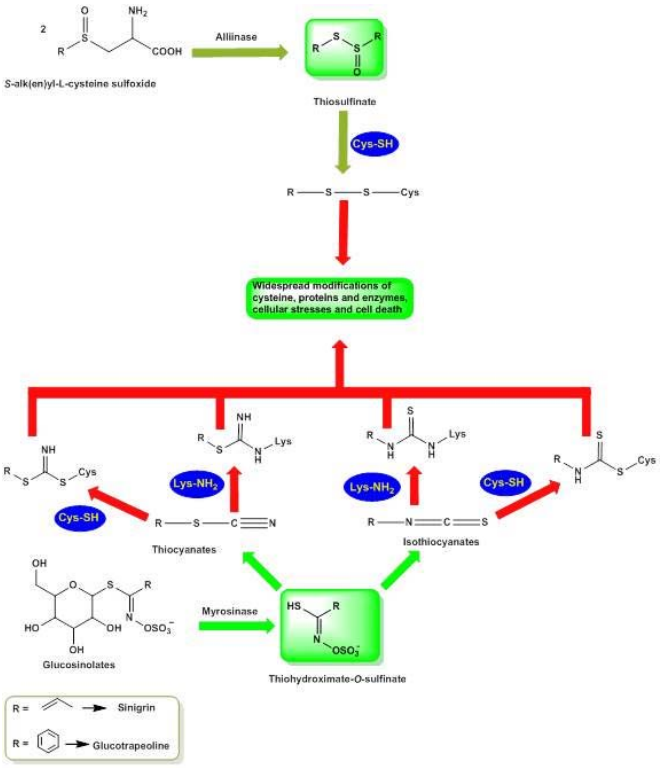

Figure 1: Enzymatic activation of otherwise virtually inactive precursors results in the formation of highly aggressive chemical species, such as thiosulfinates, isothiocyanates and thiocyanates, which readily attack different residues in key biomolecules, especially - but not exclusively - in proteins and enzymes (often GSH is also affected). This figure summarizes the "chemistry" involved in the conversion and subsequent reactions with selected biomolecules. See text for further details.

Table 1: MIC values (in µM) of each substrate/enzyme combination investigated {in the presence of 1.5 µM (66 µg/mL) of alliinase or 4.0 µM (260 µg/mL) of myrosinase, respectively}. Glucotropaeolin (GTL) was particularly active and hence was tested against *S. cerevisiae* in the presence of just 1.0 µM (65 µg/mL) of myrosinase. No activity was observed for substrates or enzymes on their own.

Strain	Alliin	PCSO	Sinigrin	GTL
Escherichia coli	100	250	750-1000	250-500
Staphylococcus carnosus	100	250	750-1000	500
Saccharomyces cerevisiae	50	100	1000	450

Since the *in situ* generation of highly aggressive allicin from alliin has been studied before by us and others, we have first turned our attention to a combination of alliinase with its various natural (alliin) and synthetic substrates (primarily PCSO). After purification and characterization of the enzyme, and the synthesis of its substrates, the most effective enzyme and substrate concentrations have been determined in *in vitro* alliinase assays based on 4-mercaptopyridine (4-MP) as indicator. Subsequently, various combinations of alliinase with its substrates were employed against bacteria, yeast and nematodes. As shown in Table 1, a combination of alliin and alliinase was particularly active against *S. cerevisiae*, with substrate concentrations required (shown as MIC values) in the range of 50 µM (alliin and enzyme alone; alliinase at 4.0 µM were not active at the concentrations employed). The allicin generating system was somewhat less active against the bacteria tested. Similarly, the artificial substrate PCSO was less efficient when compared with alliin - probably reflecting its reduced substrate properties - in line with our *in vitro* alliinase activity assays (data not shown) and also in good agreement with our recent reports [11]. The garlic-derived system was also rather effective against the nematode *S. feltiae*, reflecting the considerable activity of allicin against small parasites and the known medical and agricultural uses of this Reactive Sulfur Species (RSS) from garlic [12]. Whilst the natural substrate alliin, when applied in concentrations between 100 µM and 250 µM, was entirely inactive in the absence of alliinase, it turned highly toxic in its presence, with less than 50% of nematodes surviving after 24 h (Figure 2). Alliinase on its own, of course, was also inactive at the concentrations used. Notably, the synthetic alliin derivatives *S*-ethyl-L-cysteine sulfoxide (ECSO, the ethylanalogue), PCSO and *S*-butyl-L-cysteine sulfoxide (BCSO, the *n*-butyl-analogue) were also active in this nematode assay, yet again to a lesser extent.

The reconstituted glucosinolate/myrosinase defence system from mustard exhibits an activity similar to the one of the alliin/alliinase system. Concentrations of substrates required for activity are in the sub-millimolar range between 250 and 1000 µM, and the myrosinase concentrations required to "activate" the substrate are in the range of a few micromolar (approximately 4 µM of enzyme, 260 µg/mL of protein,). These concentrations of myrosinase were used in most studies involving bacteria and yeast, and concentrations as low as 0.04 µM, (*i.e.* 2.6 µg/mL of protein) were used in the nematode assay.

Still, there are some major differences between the two systems. The myrosinase system appears to be considerably less effective against yeast, and both sinigrin and glucotropaeolin (GTL) had to be used in ten-fold higher concentrations when compared with alliin. The mustard-derived system was also somewhat less efficient against *Staphylococcus carnosus*. In sharp contrast, a combination of GTL and myrosinase could be used to kill *E. coli*, with MIC values for the substrate in the range of 250 to 500 µM. As anticipated, myrosinase and its substrates on their own were inactive in those assays.

The "generation" of activity by myrosinase from otherwise non-toxic glucosinolates could also be observed in the nematode assay. As Figure 3 illustrates, neither substrate nor enzyme on its own exhibited any toxicity against *S. feltiae*, while a combination of both {125 µM of GTL, 0.04 - 0.08 µM (2.6 µg/mL - 5.2 µg/mL) of myrosinase} effectively killed those nematodes within 2 h of treatment, with a survival rate below 25%. Virtually no survival was observed under those conditions when the substrate concentration was raised to 250 µM. It should be noted that in our hands, GTL generally appeared to be more active than sinigrin.

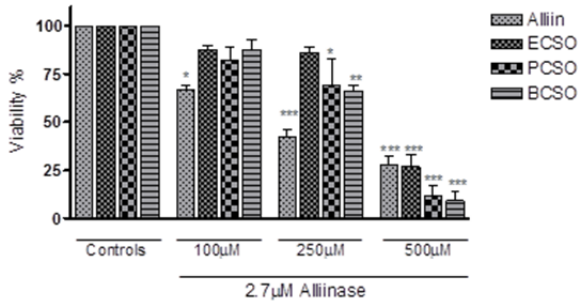

Figure 2: Use of a binary system for the substrates alliin, ECSO, PCSO and BCSO to determine nematicidal activity against *S. feltiae* in the absence and presence of 2.7 µM (132 µg/mL) of alliinase. The negative control was either enzyme or substrate alone standardized as 100 %. Significances are expressed to the negative control. Data are presented as mean (viability %) ± SD of three independent experiments, performed on three different occasions (n = 3 x 3). The parameters of statistical significance are: ns $p > 0.05$,* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Whilst so far, and in most studies elsewhere, substrate and enzyme were added more or less to the same sample and mostly within a short time interval, it is often desirable to add one component first, wait and then add the second component "later on", *i.e.* on demand and independently of the first one. Indeed, several such systems

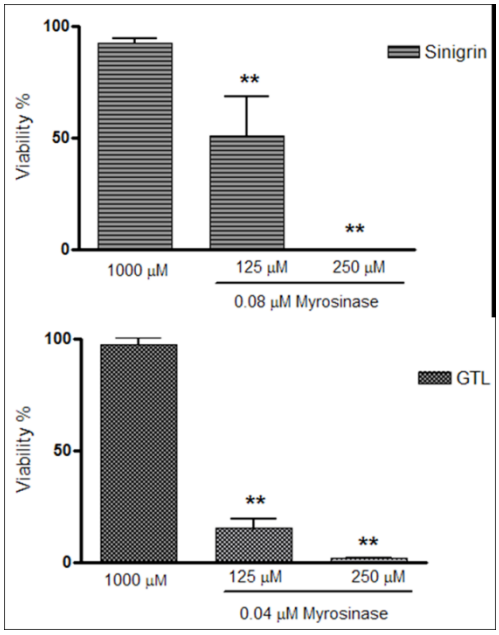

Figure 3: Viability of *S. feltiae* was reduced significantly in the presence of either myrosinase and sinigrin (upper panel) or myrosinase and GTL (lower panel) after 2 h of treatment, whilst the individual components *i.e.* enzyme and substrates on their own, were entirely nontoxic. Values represent mean (viability %) ± S.D. ns $p > 0.05$, ** $p \leq 0.01$.

have been developed for alliinase, for instance by immobilizing the enzyme on a solid support or by attaching it to antibodies [4,13]. We have, therefore, considered the possibility that either alliinase or myrosinase may attach itself to the nematodes more or less spontaneously and subsequently may serve as a local trigger for activity. Figure 4 indicates that such a sequential incubation, which is interrupted by three washing steps between adding first the enzyme and then the substrate may be possible, at least in the case of myrosinase and in this particular order. When the substrate is added after the washing steps, there is still a significant reduction of viability (below 75%). Substrate applied to "enzyme free" nematodes is not active. It appears that the myrosinase applied first had become somehow "stuck" to the organism and subsequently could be used to activate the glucosinolate at the site of the individual nematode. Here, the isothiocyanates and related compounds are no longer generated in the bulk but at the target site. It should be noted that similar experiments were also conducted in the case of alliinase, but the latter seems to wash off or lose its activity during the washing step, since no statistically significant reduction in survival could be observed when the washing step was included (Figure 4).

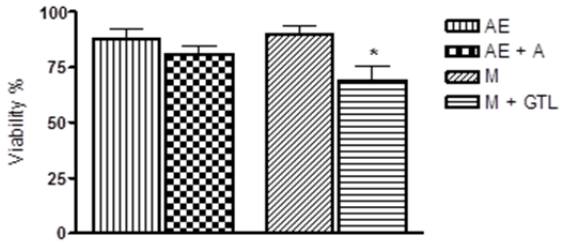

Figure 4: Incubation of *S. feltiae* with alliinase or myrosinase, followed by three washing steps and subsequent addition of the respective substrate. Viability has been determined after 24 h. Values represent mean (viability %) ± S.D. ns $p > 0.05$,* $p \leq 0.05$. See text for further details. AE: alliinase, A: alliin, M: myrosinase, GTL: glucotropaeolin.

Considering the different specificities and underlying "chemistries" observed for the alliinase and myrosinase systems, one may expect that the two systems, when employed together, may lead to a certain synergy, possibly to a double impact and greatly improved activity when compared with the two systems in isolation. We have therefore combined the two systems and created a four component "garlic flavoured mustard" system, which has been tested against *E. coli*, *S. carnosus*, *S. cerevisiae* and *S. feltiae*. The results shown in Figures 5 and 6 are complicated. Because of the complexity of such a four component system, their interpretation is not directly obvious. Nonetheless, it seems that there is no significant synergy between any of the two enzyme/substrate combinations. In some instances, it even appears that the combined systems reduce each other's activity. In the case of *E. coli*, for instance, the combination of both enzyme/substrate systems results in a reduction in cell survival, *i.e.* toxicity, which is less than the sum of the individual reductions. One may speculate that the products of the enzymatic reactions, *i.e.* the thiosulfinates, isothiocyanates and other reactive, electrophilic species, not only attack proteins in the target organism but possibly also inhibit the enzyme(s) we have employed as part of our four-component system. Those findings clearly warrant further investigation.

Our results confirm that it is possible in practice to reconstitute plant-derived substrate/enzyme systems, such as the alliinase system from garlic and the myrosinase system from mustard, with comparable ease. Whilst it is cumbersome to derive highly pure enzymes, simple purification methods, such as gel filtration, are adequate to produce both enzymes in good yields and ready to be used in such two-component systems as "activators" of antimicrobial activity. We have also seen that such enzyme/substrate combinations can be employed rather effectively against bacteria and other microbes, including more complex multicellular organisms, such as nematodes. Those results agree well with previous studies which have investigated the potential practical uses of such reconstituted systems in medicine or agriculture [9,11,13b,13d,14].

When compared with the lesser known myrosinase system from mustard and rape seeds, the garlic system seems to be particularly effective against yeast, but less so against certain bacteria and nematodes. This apparent difference in respective activity against different organisms is probably due to the special chemistry of the thiosulfinates on the one side, and the isothiocyanates and related products on the other. As already mentioned, thiosulfinates react readily, yet also practically exclusively with thiol groups in glutathione (GSH), proteins and enzymes. Among the various protein targets, the garlic-derived system therefore is fairly selective for redox sensitive cysteine proteins, which we have recently subsumed under the notation of the "cellular thiolstat" [15]. Since *S. cerevisiae* is reasonably sensitive to redox changes affecting its many reduced cysteine proteins, it is likely that such thiol-specific modifying agents hit this organism particularly hard. Unlike thiosulfinates, isothiocyanates react as aggressive and barely selective electrophiles; their targets include among others primarily amino groups, which cannot be protected by antioxidants, and modifications by isothiocyanates are usually irreversible. Hence the damage caused by those agents may be more widespread, and may also affect certain bacteria and smaller multicellular organisms.

In contrast to alliinase, myrosinase also seems to attach spontaneously to the nematodes and retain some of its activity even after washing, an aspect which bodes well for more sophisticated applications. If confirmed, these preliminary studies may provide the basis for two-component systems derived from edible plants and

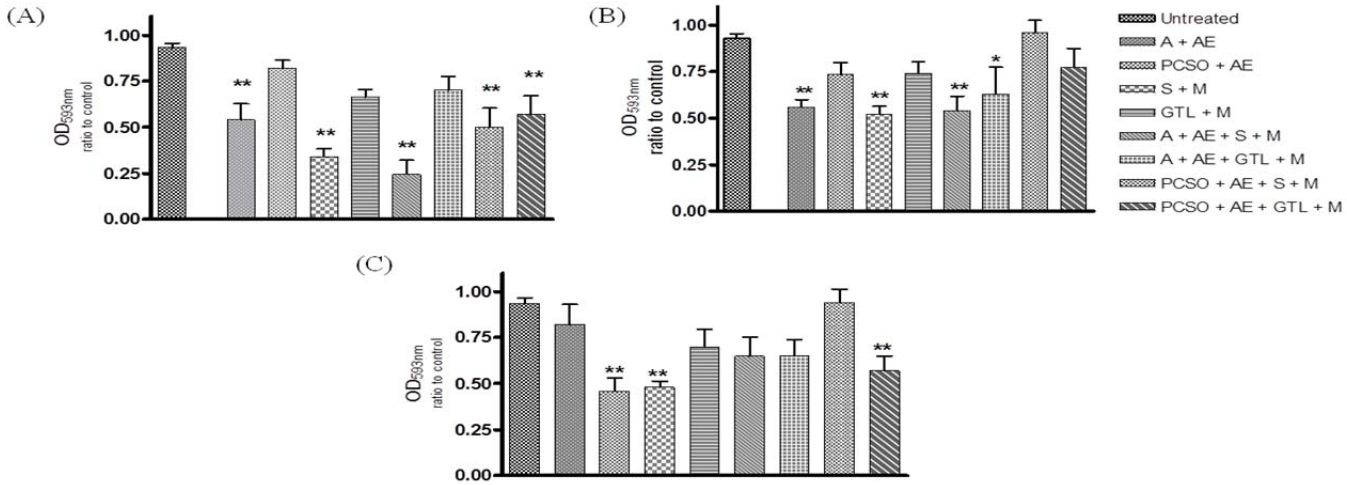

Figure 5: Towards a four-component system. Whilst each of the two systems used in isolation results in a significant reduction in viability, when employed in combination, the alliinase/substrate and myrosinase/substrate systems do not appear to develop any synergy, at least with regard to efficiency. It seems that in some instances, they may even slightly hinder each other: *E coli* **(A)**, *S carnosus* **(B)** and *S cerevisiae* **(C)**. Cell density has been measured via optical density (OD). Values represent mean ± S.D. ns $p > 0.05$ * $p < 0.05$, ** $p < 0.01$.
A: alliin, AE: alliinase, PCSO: *S*-propyl-L-cysteine sulfoxide, S: sinigrin, M: myrosinase, GTL: glucotropaeolin.

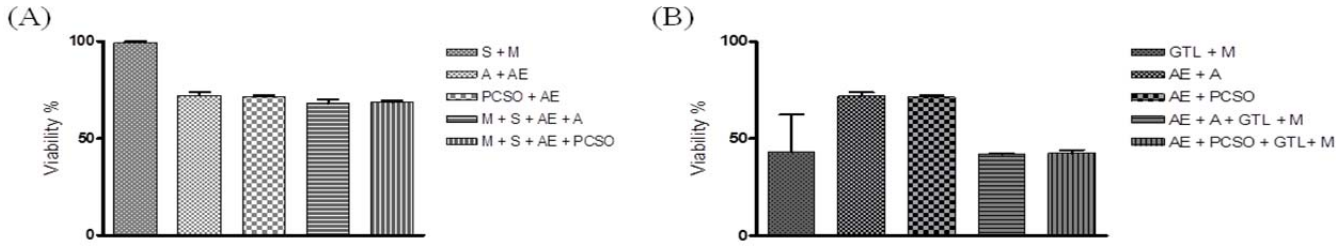

Figure 6: Toxicity of the combined systems in *S. feltiae*. Whilst each of the two systems used in isolation results in a small (alliinase/substrate and myrosinase/sinigrin) to moderate (myrosinase/GTL) reduction in viability, when employed in combination, the alliinase/substrate and myrosinase/substrate systems do not appear to develop any synergy, at least with regard to efficiency: Sinigrin (A), and GTL (B). Values represent mean (viability %) ± S.D. ns $p > 0.05$ S: sinigrin, M: myrosinase, A: alliin, AE: alliinase, PCSO: *S*-propyl-L-cysteine sulfoxide, GTL: glucotropaeolin.

with higher efficiency and selectivity. In those systems, the toxic product could be generated "on demand" and more or less exclusively at the particular site and specific location of the enzyme. Target selectivity may be enhanced further by modern delivery, immobilization, encapsulation and antibody techniques [4,13c]. An active uptake of intact, active enzyme by the target itself would even be more desirable. It would be second best to engineer genetically alliinase or myrosinase into the target or the host that requires protection.

Eventually, our studies have shown that two-component systems inspired by the phytoanticipins of edible plants are worth considering in earnest, not only in the context of a possible anti-cancer activity, but also against certain bacteria, fungi and small parasites. Intriguingly, nature provides numerous such substrate/enzyme systems, and it seems that they differ in their activity profiles. It is therefore worth exploring several of those systems further. Since individual components of these systems, such as enzyme or substrate on its own, are non-toxic, the application of such two-component systems is therefore considerably safer, more selective and target specific when compared with inherently toxic agents. Future studies need to investigate the efficient production and stabilization of the enzymes as well as the effective individual "delivery" of both enzymes and substrates in a medical or agricultural context. Although it has not been possible to demonstrate a direct synergy in the four-component "garlic flavoured mustard" system with regard to efficiency against one target, combinations of two or more enzyme / substrate pairs may still hold some promise, for instance by providing a wider spectrum of activity against different targets, assuming the systems do not inhibit - or otherwise interfere with - each other. Eventually, the availability and ease of preparation of both the enzymes and their substrates, and the low concentrations required for activity, especially in the case of the enzymes, turn those systems into an effective and selective alternative to chemically synthesized artificial drugs or phytoprotectants.

Experimental

Materials: Chemicals were mostly purchased from Sigma-Aldrich Chemie GmbH (Steinheim Germany). L-cysteine hydrochloride was purchased from Applichem GmbH (Darmstadt Germany), and Con A Sepharose ^{TM} 4B and the gel filtration columns from GE Healthcare Bio-science AB Uppsala, Sweden, whilst glucotropaeolin tetramethylammonium salt (GTL) was donated by the Plant Breeding and Acclimatisation Institute in Poland. MilliQ water (resistance ≥ 18 MΩ/cm) was used for the biological studies. The substrates of alliinase, *i.e.* alliin, ECSO, PCSO and BCSO were synthesized from L-cysteine as racemic mixtures according to the procedure of Stoll and Seebeck with minor modifications [16 ,17]. Analytical results (^{1}H NMR, ^{13}C NMR, IR spectra and melting points) of all compounds synthesized were in accordance with literature values. *Steinernema feltiae* was obtained from Sauter and Stepper (Ammerbruch, Germany).

Isolation of enzymes: The two enzymes used as part of this study, *i.e.* alliinase and myrosinase, were isolated from garlic cloves and

mustard seeds, respectively. Both enzymes were partially purified with the intention to obtain preparations being useful for practical applications. In brief, alliinase was extracted from 40 g of freshly peeled commercially available garlic cloves according to the literature procedure, with minor modifications [18]. Enzyme purity was determined by native SDS PAGE electrophoresis, the concentration in the individual fractions was determined by the Bradford assay, and the activity confirmed in a standard spectrometric activity assay [19]. Myrosinase was extracted from the seeds of *Sinapis alba* obtained from a local farm (Wingertsweiherhof, D-*66564* Ottweiler, Saarland) according to a modified literature procedure [20]. The concentration of protein in each sample was determined with a standard Bradford assay. The activity of myrosinase was estimated using the spectrophotometric method described by Piekarska *et al.* [21], which is based on measuring the enzymatic decomposition of either sinigrin or glucotropaeolin. In both cases, *i.e.* for alliinase and for myrosinase, enzyme purity was well above 75 % of total protein. It should be noted that further purification of both enzymes is, in theory, possible, but labor-intensive and hence not realistic if such systems are being used for practical applications. In our studies, the enzyme preparations on their own were always used as "controls" to rule out any undesired non-specific activities caused by any of the truly minor contaminating proteins.

General procedures: All experiments were performed at room temperature. For kinetic studies, UV/VIS spectra were recorded using a Cary50*Bio* Spectrophotometer. NMR spectra (in D_2O) were recorded on a Bruker (Rheinstetten, Germany) Type DRX 500 and Avance 500 instruments (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR). Liquid chromatography-mass spectrometry (LC-MS) was performed on a Bischoff Lambda instrument using a YMCC-18 pro-column and methanol/water as mobile phase (flow rate 1.0 mL/min). IR spectra were recorded using a Frontier FTIR spectrophotometer (Perkin Elmer LAS GmbH Rodgau, Germany). Melting points were determined by using Electrothermal's Digital Memory Melting Point Apparatus IA 9300.

Antimicrobial activity assays involving *Escherichia coli, Staphylococcus carnosus* and *Saccharomyces cerevisiae:* As indicator for activity against bacteria and fungi, Minimum Inhibitory Concentrations (MIC) were determined using the broth microdilution method [22]. For this purpose, cultures of *Escherichia coli* K2 and *Staphylococcus carnosus* TM300 were placed on Luria-Bertani medium (LB medium) and *Saccharomyces cerevisiae* on Yeast Extract-Peptone-Dextrose medium (YPD medium) at 37°C overnight. Inocula were prepared by suspending colonies of these cultures in sterile 0.85% NaCl solution, adjusted to 0.5 on the McFarland scale (1-5 x 10^8 CFU/mL for bacteria and 1-5 x 10^6 CFU/mL for yeast). Substrates were diluted in LB or YPD broth at different concentrations and aliquoted to each well of a 96-well plate and mixed with 10 µL of the bacterial suspension. As a final step, isolated alliinase and / or myrosinase (0.5 µM to 4.0 µM, *i.e.* 24.5 µg/mL to 260 µg/mL) were added and the plates were incubated at 37°C for 24-48 h. MIC was defined as the lowest concentration that inhibited visible growth. The assay was conducted in triplicate and on three separate occasions (3x3). A mixture of penicillin, streptomycin and amphotericin (4 U, 0.4 µg/mL and 1 µg/mL, respectively) was used as reference antibiotic control, inhibiting microbial growth by 100%.

For the combination assays, bacterial and yeast growth was assessed by measuring the optical density (OD) at 593 nm after 24 h incubation using a microplate reader. Concentrations of substrates and enzyme above the MIC previously determined were chosen as follows: for bacteria 0.75 µM (33 µg/mL) of alliinase, 50 µM of alliin or PCSO, 2.2 µM (143 µg/mL) of myrosinase and 50 or 100 µM of sinigrin or GTL; for yeast, 0.75 µM (33 µg/mL) of alliinase, 25 µM of alliin, 50 µM of PCSO, 1 µM (65 µg/mL) of myrosinase and 100 µM of either sinigrin or GTL. Three repeats were performed for each combination. Results are expressed as mean ± standard deviation (SD). The statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparison test. A value of $p \leq 0.05$ is considered statistically significant.

Nematicidal activity assays involving *Steinernema feltiae:* The assay indicative of nematicidal activity was conducted using the nematode *Steinernema feltiae* as a model organism according to procedures described previously by Schneider *et al.* and Czepukojc *et al.* [23]. Briefly, aliquots of a suspension containing nematodes were placed in each well of a 96-well plate and the viability was determined, after which substrate and enzyme were added. To establish if the substrate can be applied independently from and well after incubation with enzyme, nematodes were incubated with either alliinase (2.7 µM, *i.e.* 132 µg/mL) or myrosinase (0.08 µM, *i.e.* 5.2 µg/mL) for 1 h, washed 3 times with PBS and plated before the respective substrates were added at a concentration of 1000 µM. All the plates were kept in the dark, at room temperature for 24 h, at which point viability was checked.

Three independent experiments for each assay were carried out on 3 separate occasions and results are expressed as mean ± standard deviation (SD). The statistical significance was calculated as above and a value of $p \leq 0.05$ is considered statistically significant.

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List of abbreviations: A, alliin; AE, alliinase; BCSO, *S*-butyl-L-cysteine sulfoxide; CFU, Colony Forming Units; ECSO, *S*-ethyl-L-cysteine sulfoxide; GSH, glutathione (reduced form); GTL, glucotropaeolin; LB, Luria-Bertani; M, myrosinase; MIC, Minimum Inhibitory Concentration; OD, Optical Density; PCSO, *S*-propyl-L-cysteine sulfoxide; RSS, Reactive Sulfur Species; S, sinigrin; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; YPD, Yeast Peptone Dextrose.

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