Myrosinase activity in microorganisms and its possible health benefits for humans
&
Potential antibacterial effect of marine biological waste products

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Uppsala 2009

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Myrosinasaktivitet hos mikroorganismer och dess potentiellt hälsofrämjande effekt hos människor
&
Potentiell antibakteriell effekt av marina biologiska restprodukter

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Abstract

Cancer is an illness that affects countless persons, either they themselves are afflicted or someone in their vicinity is. Research has shown that when glucosinolates – secondary metabolites in plants from the Brassicaceae family – are degraded in the stomach of animals via the enzyme (myrosinase), their metabolites can counteract the formation of tumour cells. However, the natural degradation in the stomach might not be sufficient, so it has been proposed that intake of microbes with myrosinase activity might be beneficial for the degradation of glucosinolates. This study was therefore conducted to search for microbes with myrosinase activity that might be safely digested.

Chosen bacteria were first screened for glucosinolate breakdown based on a barium sulfate test which was inconclusive due to difficulties to discern the precipitate. Three bacterial strains were thought to be indicative of precipitation and were incubated with either sinigrin or mustard extract. Glucose was measured as an indicator for how much glucosinolate had been degraded during the incubation. Due to inconsistency between the results it was not possible to deduce whether glucosinolates were degraded or if the bacteria themselves produced glucose via an alternative pathway.

Two strains of fungi were also tested for glucosinolate degradation using the barium sulfate screen containing sinigrin or vegetative extracts. *L. maculans* grew only on medium containing sinigrin while Botrytis grew indiscriminately on all media including the negative control.

Due to the difficulties in attaining accurate results it would be wise to try to achieve more exact measuring methods for myrosinase activity should this research venue be continued.

Resistance to antibiotics is a growing problem today that may cause havoc in the treatment of bacteria in the future. To solve this problem researchers have started looking towards marine organisms for antibacterial substances. The reason for the specific interest in marine organisms is that they represent a large biodiversity and have evolved separately from terrestrial animals for billions of years and should thus have novel molecules that could be used on new targets on bacteria. In this paper two samples from marine food processing were tested against several common microbes to see if they had any inhibitory effect.

The samples were either filtrated or heat inactivated before screening against bacteria using an agar diffusion assay along with positive controls and untreated samples. The samples demonstrated an inhibitory effect against most of the chosen bacteria but it was thought to be of biological nature rather than chemical since the inhibition was only discerned when untreated samples were used on the bacteria plates. Two fungal strains were also tested but no inhibition was found when screened with the samples whether they were filtrated or not.

The marine samples should be further tested since some inhibitory action against the bacteria could be found, but first it should be determined whether it is because of a new molecule or an already known structure.

Sammanfattning

Cancer är en sjukdom som påverkar många människor, antingen är de själva sjuka eller känner någon som drabbats. Forskning har visat att när glukosinolater – sekundära metaboliter hos växter tillhörande Brassicaceae – bryts ner i magen på försöksdjur via ett enzym (myrosinas) kan deras metaboliter ha en inhibitorisk effekt på uppkomst av tumörceller. Den naturliga nedbrytningen kan dock vara för låg och därför har det föreslagits att intag av mikrober med myrosinas aktivitet kan vara gynnsam för nedbrytningen av glukosinolater. Denna studie utfördes därför för att finna mikrober med myrosinas aktivitet som kan ätas utan risk.

Utvalda bakterier screenades för nedbrytning av glukosinolater via ett barium sulfat test vilket inte gav klara resultat pga. svårigheter att urskilja urfällning. Tre stammar förmodades dock kunna vara positiva för urfällning och inkuberades med sinigrin eller senapsextrakt. Glukoshalten mättes som en indikator för hur mycket glukosinolater som hade brutits ned under inkubationen. Variationer mellan resultaten gjorde det inte möjligt att påvisa om uppkomsten av glukos var pga. myrosinasaktivitet eller om bakterierna själva producerade glukos via en alternativ väg.

Två svamparter testades även de för myrosinasaktivitet på medier innehållandes sinigrin eller växtextrakt via bariumsulfat testet. *L. maculans* växte enbart på medium som innehöll sinigrin medan *Botrytis* växte urskiljningslöst på alla medier inklusive den negativa kontrollen.

Ska denna forskning fortgå vore det klokt att försöka hitta effektivare och mer exakta sätt att mäta myrosinasaktivitet eftersom det var svårt att få precisa resultat med metoderna i denna studie.

Resistens mot antibiotika är ett växande problem idag som kan komma att orsaka problem i kampen mot bakterier i framtiden. För att lösa detta problem har forskare börjat söka efter antibakteriella ämnen hos marinlevande organismer. Anledningen till detta är att de har stor biodiversitet och har utvecklats separat från marklevande djur under miljarder år och borde därmed ha många oupptäckta molekyler som kan användas för att skapa nya antibakteriella måltavlor hos bakterier. I detta projekt screenades två marina provämnen mot flera vanliga mikrober för att undersöka om de hade en inhibitorisk effekt mot dem.

Proven antingen filtrerades eller värmeinaktiverades innan de screenades via ett agar diffusions test mot bakterierna tillsammans med positiva kontroller och även obehandlade prov. Proven hade inhibitorisk effekt mot de flesta av bakterierna men antogs mer bero på någon biologisk komponent snarare än en kemisk eftersom inhibitionen endast uppstod när obehandlade prov användes. Två svampar testades även de, men proverna hade ingen effekt mot dem, vare sig de var filtrerade eller inte.

Proverna borde fortsätta testas eftersom de utövade någon sorts inhibition mot bakterierna, men det borde först fastställas om inhibitionen är pga. en redan känd molekyl eller om det är en helt ny struktur.

Table of contents

Introduction

Glucosinolates and Myrosinase

Glucosinolates are organic compounds rich in sulfur that consist of three parts, a glucose, a sulfonated oxime group and a side chain that differs for each glucosinolate (Fig. 1). The side chain is derived from an amino acid and the glucosinolates are ordered into three major groups (aliphatic, aromatic or indolylic) dependent on which amino acid is used. The amino acids belonging to the aliphatic group are Methionine, Leucine, Alanine, Isoleucine and Valine while Phenylalanine and Tyrosine are aromatic and Tryptophane belongs to the indolyl group (Halkier et al. 2006).

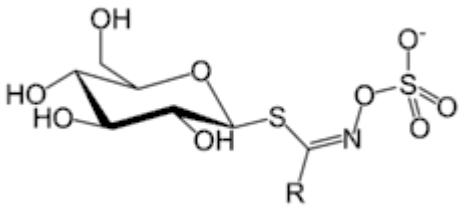

Fig. 1 Common structure of glucosinolates with the side chain (R) that varies for each glucosinolate. (Wikipedia)

The glucosinolates are mainly observed in plants of the family Brassicaceae in the order Brassicales where they occur as secondary metabolites and are a major constituent in the plant's protection against herbivores. When attacked the plant releases the glucosinolates which are subsequently hydrolyzed by myrosinase, a thioglucohydrolase (EC3.2.1.147), thus producing metabolites that can either be toxic to the assailant, simply deterring due to bad taste or smell or they can attract natural enemies that are predatory against the plant assailant (Rask et al. 2000, Mikkelsen et. al. 2001).

Biosynthesis of Glucosinolates

Glucosinolates are synthesized in three steps; chain elongation, core synthesis and secondary modifications. The core synthesis is the same for all glucosinolates while chain elongation occurs only for certain amino acids and the secondary modifications vary for each glucosinolate (Grubb & Abel 2006). This gives rise to an extensive diversity of glucosinolates with >120 different structures known today (Fahey et al. 2001).

The chain elongation starts by deamination of the amino acid thus producing an α-keto acid, which further reacts with acetyl-CoA to form a 2-malate derivative. This structure is further processed via isomerization to produce a 3-malate derivative that is elongated by a methylene through oxidative decarboxylation. The resulting elongated α-keto acid can be further extended by another round of the three-step sequence to gain an additional methylene or it can be transaminated and then proceed to the next step, core synthesis (Fig. 2). The side chain can be elongated up to nine times before it proceeds to core synthesis (Mikkelsen et al. 2001, Wittstock & Halkier 2002, Halkier et al. 2006).

In the core synthesis the amino acid (either from the elongation route or non-processed) is converted to an aldoxime by oxidation by cytochrome P450s of the CYP79 family, then the aldoxime is oxidized by cytochrome P450s in the CYP83 family to produce an highly unstable intermediate, either an *aci*-nitro compound or a nitrile oxide. These compounds then spontaneously react with thiols (probably from cysteine) to produce an *S*-alkyl thiohydroximate. This conjugation is believed to be enzymatically controlled by a glutathione-*S*-

transferase-like enzyme. Next the S-Alkyl thiohydroximate is cleaved by C-S lyase to form a thiohydroximic acid which is then glucosylated by

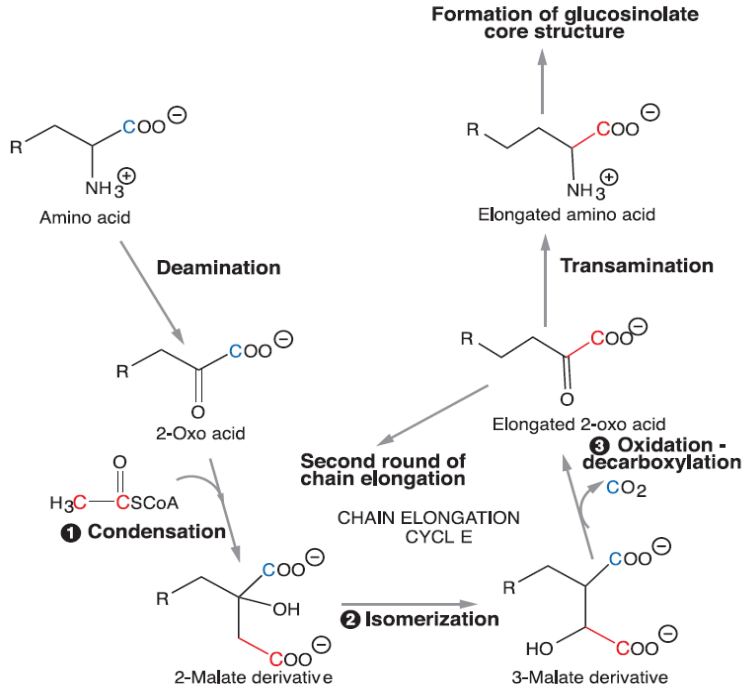

Fig. 2 Side chain elongation.

The amino acid is deaminated to produce a 2-oxo acid which is condensed with acetyl-CoA to produce a 2-Malate derivative that is isomerized to generate a 3-Malate derivative. The 3-Malate derivative undergoes oxidation-decarboxylation that adds a methylene and thus creates an extended 2-oxo acid that can either undergo further elongation or be transaminated to start core synthesis. The carbons in red are the ones contributed by the acetyl-CoA and the ones in blue are from the original COO^- (Halkier et al. 2006).

thiohydroximate glucosyltransferase to produce a desulfoglucosinolate that in turn is sulfated by desulfoglucosinolate sulfotransferase to form a glucosinolate (Fig. 3) (Mikkelsen et al 2002, Halkier et al. 2006, Grubb et al. 2006).

The parent glucosinolate can be further altered by secondary modifications that occur on the side chain. These modifications are crucial since they determine the nature of the products formed after degradation of the glucosinolate by the myrosinase enzyme (Halkier et al. 2006). The side chains can undergo oxidation, elimination, alkylation, esterification, hydroxylation, methoxylation, desaturation or glycosylation (Mikkelsen et al. 2001, Grubb et al. 2006, Halkier et al. 2006, Rask et al. 2000). Especially side chains derived from methionine are prone to undergo further modifications (Halkier et al. 2006, Grubb et al. 2006).

Degradation

Upon hydrolysis by the myrosinase the glucosinolate forms glucose and an unstable intermediate, thiohydroximate-*O*-sulfate. This intermediate then undergoes further transformation to yield the active metabolite; isothiocyanate, nitrile, thiocyanate, epithionitrile, oxazolidine-2-thione or other less common products (Fig. 4.). The structure of the final product(s) is dependent on chain elongation, secondary modifications, myrosinase interacting proteins (e.g. epithiospecifier proteins (ESP)), co-factors and physical circumstances such as pH (Grubb et al. 2006, Rask et al. 2000, Halkier et al. 2006, Bones & Rossiter 2006).

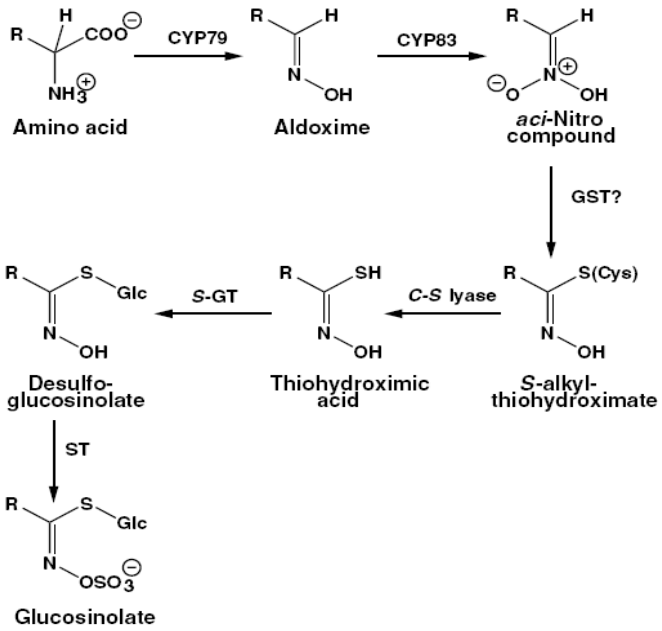

Fig. 3 Core synthesis of glucosinolates.

The amino acid is oxidized to an aldoxime by CYP79 enzymes. The aldoxime is then further oxidized to *S*-Alkyl thiohydroximate by enzymes in the CYP83 family. The *S*-Alkyl thiohydroximate is cleaved by C-S lyase before being glucosylated and sulfated (Halkier & Gershenzon 2006).

Anti-carcinogenic effect

For several years it has been shown that certain glucosinolate metabolites have an anticarcinogenic effect on animals and humans. Various mechanisms for this effect have been proposed and they mostly involve isothiocyanates. Certain isothiocyanates can lower the risk for cancer by inducing phase 2 enzymes, which neutralize reactive compounds that might otherwise cause mutagenesis (Verhoeven et al. 1997, Talalay & Fahey 2001, Keum et al. 2004). It has as well been shown that allyl isothiocyanate that originates from sinigrin can reduce metastasis by inhibiting metallo proteinases. The metallo proteinases degrade the extracellular matrix (ECM), which is the first step in the process of metastasis (Hwang & Lee 2006). Isothiocyanates can also induce apoptosis, e.g. phenethyl isothiocyanate induces c-Jun N-terminal kinase, which has a major role in apoptosis caused by e.g. mutagenic substances (Keum et al. 2004).

Most of the anticarcinogenic effect of glucosinolates is attributed to their metabolites, however the majority of humans eat their vegetables cooked and during the heating the myrosinase responsible for the degradation is inactivated. It has been reported that intestinal micro flora in humans have myrosinase activity, but it would be beneficial if additional myrosinase-containing bacteria could be taken separately to enhance the production of glucosinolate metabolites in the intestines.

In this study several bacteria reported to have degraded glucosinolates were re-tested for myrosinase activity; *Lactobacillus agilis* R16 (Palop et al. 1995), *Bifidobacterium pseudocatenulatum*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* (Cheng et al. 2004) and *Bacteriodes Thetaiotaomicron* (Elfoul et al. 2001). Several previously untested strains of Lactobacilli (listed in material and methods) were also screened for myrosinase activity.

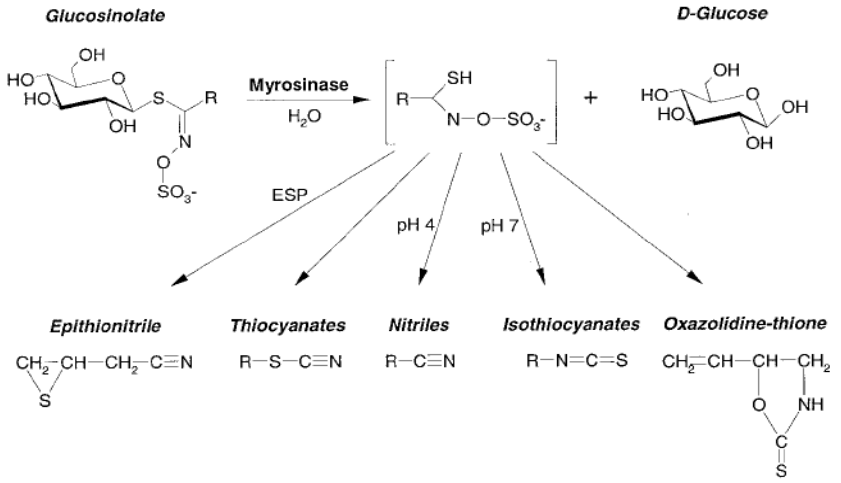

Fig. 4 General outline of glucosinolate degradation. (Rask et al. 2000)

Marine biological waste products from food processing

In today's world the over-consumption of antibiotics cause an escalating problem of multi-drug resistant bacteria e.g. *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

S. aureus was first treated with penicillin until resistance was reported in the 1940s. The penicillin resistant strain soon invaded the world and caused huge problems due to its strong virulence and high mortality. In 1960, meticillin was introduced to the market and it was very effective against the penicillin resistant *S. aureus* strain. Nonetheless, already in 1967 multi-drug resistant MRSA (meticillin resistant *S. aureus*) started to appear and in 1990s 22 epidemic MRSA strains were identified (Shito 2006, Grundmann et al. 2006). Due to the resistances glycopeptides such as vancomycin have been the main treatment against MRSA. However *S. aureus* strains resistant against vancomycin has been found during the last couple of years (Shito 2006, Grundmann et al. 2006, Beović 2006).

Another bacterium that is known for its antibiotic resistance is *P. aeruginosa*. The main constituent that enables *P. aeruginosa's* multi-drug resistance is its capability to produce a wide variety of β-lactamases (Bonomo & Szabo 2006) and it may be resistant to all available antibiotics (Beović 2006).

Existing antibiotics focus primarily on four targets in bacteria; disruption of the cell wall, interference with enzymes essential for the bacteria, inhibition of DNA synthesis or obstruction of ribosomal activity (Murray et al. pp 204-205, Brown & Wright 2005). To overcome the effects of antibiotics bacteria employ five strategies; modification of the target site, a lesser intake of the antibiotic by reduced permeability of the cell, excretion of the antibiotic by efflux pumps, inactivation of the antibiotic or alterations in the cell to produce resistant metabolic pathways (Murray et al. p. 720). The need for new antibiotic targets is urgent; however, there are certain preferences that an antibiotic target should fulfill to be worth pursuing; it should not be present in human cells, it should preferably be an enzyme with a well known function and the target should be vital for survival (Brown & Wright 2005).

The arising multi-drug resistant bacteria highlight the need for other antibacterial substances. Today approaches have been made to discover new antibacterial material from extreme environments such as hot springs or from

oceans instead of the traditional usage of soil bacteria or development of synthetic antibiotic molecules.

Reasons for this focus on the marine environment are that the organisms there have an extensive biodiversity (Sogin et al. 2006, Bhadury et al. 2006) – due to large variations in their living conditions (Kennedy et al. 2008, Bhadury et al. 2006) – and have evolved separately from terrestrial animals for 3,5 billions of years (Sogin et al. 2006) and should thus have novel biomolecules and pathways (Kennedy et al. 2008, Bhadury et al. 2006, Salomon et al 2004). Terrestrial microorganisms might be sensitive to these marine biomolecules since they would not have encountered them before and thus not have a self-defense against them.

In this study two substances, which have shown positive signs of being antibacterial in field studies (not published), derived from marine food processing ("biological waste products") and obtained from Lars Forsén (Glanshammar), were screened against four different human pathogenic bacteria and two fungal strains to observe potential antimicrobic effect.

Aim of the present study

- To measure glucosinolate concentration in extracts of mustard seeds and cabbage
- To identify microorganisms with myrosinase activity
- To identify possible antimicrobial effects of marine biological waste products from food processing on various bacterial strains and fungi

Material and methods

Glucosinolates and myrosinase

Media

Media used for growth or screening of bacteria were MRS with agar and glucose (MRS) or without them (MRS-A-G) or with either one (MRS-G or MRS-A) (10g Casamino acids, 10 g beef extract, 5 g yeast extract, 1 g Tween-80, 2 g K_2HPO_4, 5 g sodium acetate, 1.33 g tri-ammonium citrate, 0.2g magnesium sulfate·$7H_2O$, 0.05g manganese sulfate · H_2O, 20g D(+)-glucose, 15g agar and milliqQ water (MQ H_2O) to a final volume of 1L), PDA(Duchefa Biochem), PDB, Barium medium (5 mM ammonium chloride, 2.5 mM barium chloride, 3.15 g agar and MQ H_2O to a final volume of 200 mL), BHI-agar (47 g BHI agar (Oxoid), 5 g yeast extract and MQ H_2O to a final volume of 1L. 2.5 mg haemin was added to half of the medium before plating), BHI-broth (37 g BHI-broth, 5 g yeast extract, 5 mg haemin and MQ H_2O to a final volume of 1L) and MRS without glucose, with agar and with 0.2 g magnesium chloride·$6H_2O$ instead of magnesium sulfate and 0.05 g manganese chloride instead of manganese sulfate (will be called MRS-G-SO_4).

Glucosinolate extract from mustard seeds

At four different times 200 g of mustard seeds (Maxi) were ground, 50 g at a time for 2×10 s with 3 pulses (each 1s) in between, in a small blender/coffee grinder, poured into 500 mL of boiling methanol (99.8 %) and boiled for 5 min. The following steps varied slightly for each time: the first mixture was left to cool for 40 min whereupon all methanol had evaporated and an additional amount of 300 mL of boiling methanol was added before filtration through a Munktell filter paper; the second mixture was only left to cool for 5 min before proceeding with filtration so no additional methanol was added; from the third mixture most of the methanol had been taken up by the seeds or evaporated after a cooling period of 20 min so 350mL of boiling methanol was added before filtration; when the fourth mixture was left to cool (10 min) it was covered in saran wrap to avoid evaporation so no additional methanol was added before the filtration. The methanol was evaporated by Rotavapor and the filtrate was then rehydrated with 0.02 M Sodium acetate, pH 5. Here, there were again some variations in the procedures, filtrates one and two were pooled together and no further purifications were made before separation on ion exchange columns. Filtrate three was further purified by centrifugation (10,000 rpm (16,500 *g*) for 20 min at 12°C and 2× 20,000 rpm (50,300 *g*) for 10 min at 4°C) as was filtrate four (11,749 rpm (16,500 *g*) for 20 min at 4°C) before being filtrated once again with Munktell filter paper and also 0.45 and 0.2 µm Millipore filters. All filtrates were stored air tight at 4°C in darkness.

Glucosinolate extract from cabbage

A small cabbage head was cut into 1×1 cm pieces after removal of the outer leaves, left out in room temperature for ~72 hours, crushed with 2 L of boiling methanol (99. 8%) for 20 seconds in a blender, boiled for an additional 5 min and filtrated through a Munktell filter paper before removal of the methanol from the filtrate by rotavapor.

Glucosinolate extracts from various strains of cress

Glucosinolate extracts from different kinds of cress from previous experiments were also used. The extracts were made using the same method as in the cabbage extraction. The cresses used and the glucosinolate concentrations were; Indian cress (roots) in methanol, 2.89µg/mL, Watercress (roots) in methanol, 1.635µg/mL, Wintercress (shoots) in methanol 5.985µg/mL and Tindora (roots) in methanol 1.431µg/mL.

Ion exchange chromatography

The ion exchange gel was prepared by adding Sephadex (A-25) to an excess of 0.5 M Sodium acetate, pH 5. The solution was filtered through a Munktell filter paper and resuspended in 0.5 M Sodium acetate, pH 5. This was repeated once more (without resuspending the last time) before the gel was washed with MQ water and resuspended in twice the gel volume of 0.02 M Sodium acetate, pH 5. The columns were made by putting glass fiber at the bottom of 50 mL syringes and adding ~35 mL of the Sephadex.

The glucosinolate filtrates (mustard and cabbage) were added to the columns and the bound glucosinolates were eluted by the addition of methanol (60 %). Both flow through and elution fractions were stored at 4°C in dark for further analysis using spectrophotometry.

Measurement of glucose concentration in glucosinolate extracts

When glucose concentration was measured in mustard (pooled filtrates one and two) and cabbage, samples were taken from both flow through and elution and diluted with 0.02 M sodium acetate, pH 5 or methanol (60 %) respectively to concentrations of 100 %, 75 %, 50 % and 25 %. From each concentration 5×62 µL was taken and to four of them Myrosinase (0.5 µL myrosinase in 24.5 µL 50 mM sodium citrate, pH 4.5) was added, while the fifth was kept as a blank and to which only 25 µL of 50 mM sodium citrate was added. The samples were incubated for 2 h at 37°C before being heat inactivated at 95°C for 5 min. Two series of glucose standards were prepared with either solvent i.e. 0.02 M Na-acetate or methanol (60 %). The glucose standard (Randox) contained 5.55 nmol/µL of glucose and 2, 3, 5, 10, 15 and 20 µL of the standard were diluted with either solvent to a final volume of 87 µL while a negative control was also prepared consisting of pure solvent. 250 µL of glucose test reagent was added to all samples and standards which were then incubated for 15 min at 37°C before absorbance at 550 nm was measured.

Two concentration time lines were made, the first using a different concentration for each fraction (cabbage flow through 75 %, cabbage elute 75 %, mustard flow through 25 % and mustard elute 50 %) and incubation times of 2 h, 4 h and 65 h while the other used a concentration of 10 % for all fractions and shorter incubation times, 0.5 h, 1 h, 2 h, 4 h and 24 h. Concentration time lines were also made for filtrate three and four (mustard) with a concentration of 10 % for all fractions and incubation times of either 0.5 h, 1 h, 2 h, and 4 h (filtrate three) or 0.5 h, 1 h, 2 h, and 3 h (filtrate four).

Screening for glucosinolate breakdown

Plates containing 6 mL of Barium medium and either 5.78 µg Indian cress root in methanol, 3.27 µg Water cress root in methanol, 11.97 µg winter cress shoot in methanol, 2.863 µg Tindora shoot in methanol or 5 mM purified sinigrin from

horseradish were cast plus a negative control containing only barium medium. Upon these plates 5 µL of *Leptosphaeria maculans* (Leroy) spores in water were spread and incubated using a regime of 22°C in light 16 h, and 16°C in dark 8h for six days.

L. agilis R16 (TNO, MTA) colonies grown on MRS-G plates were spread upon plates, containing either only MRS-G-SO_4 or MRS-G-SO_4 with 2.5 mM barium chloride or MRS-G-SO_4 with 2.5 mM barium chloride and 5 mM sinigrin, and incubated at 28°C for three days.

Plates containing MRS-G-SO_4 with 2.5 mM barium chloride and different concentrations of sinigrin (5 mM, 1 mM and 0.25 mM) plus two negative controls containing MRS-G-SO_4 with or without 2.5 mM barium chloride were used for screening *B. thetaiotaomicron* (RIKEN JCM no 5827), *B. pseudocatenulatum* (RIKEN JCM no 7040), *B. adolescentis* (RIKEN JCM no 7045), *B. longum* (RIKEN JCM no 7050), *Lactobacillus Johnsonii* (SLU 1.30), *Lactobacillus coryniformis* (SLU M333 14147), *Lactobacillus plantarum* (SLU H92:3), *Lactobacillus saeri…neri* (SLU GDA 154), *Lactobacillus mucosae* (SLU S32), *Lactobacillus reuteri* (SLU DSM 17938), *Lactobacillus reuteri* (SLU MM4-1A), *Lactobacillus acidophilus* schaedler (SLU 5.89), *Lactobacillus salivarius* (SLU 5.83), *Lactobacillus murimase*-like (SLU Na3 2.34), *Lactobacillus salivarius* schaedler (SLU 5.90), *Lactobacillus* "new specie rat" (SLU Na1 1:43) and *Lactobacillus* "new specie 2 rat" (SLU 2:38). Before the screening the *B. thetaiotaomicron* and the Bifidobacteria were cultured anaerobically on BHI-plates at 37 °C. The *B. thetaiotaomicron*, the Bifidobacteria and the Lactobacilli were grown at 37°C for a week. While the Lactobacilli were grown both anaerobically and aerobically the *B. thetaiotaomicron* and the Bifidobacteria were only grown anaerobically.

Botrytis was screened for myrosinase activity by spreading 5 µL of spores in water on plates containing pure barium medium (neg. ctrl) and on plates also containing either 5 mM sinigrin, 1.3 mL extract (mustard flow through or elution (filtrate one and two), Indian krasse, Vattenkrasse or Vinterkrasse) per 4 mL medium or 0.5 mL Tindora extract per 1.5 mL medium. The plates were incubated using regime of 22°C in light 16h and 16°C in dark 8h.

L. maculans (Leroy) was screened with the same substances and concentrations as Botrytis except for the Tindora extract.

100 µL of *L. Johnsonii*, *L. plantarum*, *L.* "new strain 2 rat" and *L. agilis* R16 were inoculated, in 10 mL of MRS-G (neg. ctrl) and MRS-G containing 1 mM sinigrin, and incubated at 37°C on a circular shaker (150-220 rpm) for 2 h, 6 h, 24 h and 48 h. The samples taken at the different time points were spun down (13,000 rpm for 10 min) and the supernatant saved. The glucose content was measured for each sample by adding glucose test reagent, incubating for 15 min at 37°C and then measuring the absorbance at 550 nm as described above. The bacteria were also inoculated in MRS-G containing 1 mM flow through fraction from filtrate four (the flow through had first been freeze dried and resuspended in MQ H_2O). Samples were taken at 2 h, 6 h and 24 h and processed and measured as before, but this time the pellets from 6 h and 24 h were saved and a viable count was made. The pellets were rehydrated with MQ H_2O and diluted to four concentrations, 100 %, 10 %, 1 % and 0.1 % which were spread on MRS plates and incubated over night at 37°C.

Antibacterial effects of marine biological waste products from food processing

Growth media

Nutrient agar, LB medium with and without agar (10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar and MQ H_2O to a final volume of 1L) and blood agar were used for growth and screening.

Marine biological waste products

Samples from the marine biological waste products obtained from food processing industry were filtered through Munktell filter paper, 0.45 μm and 0.2 μm Millipore filters, then spread on MRS, BHI and LB plates to check for bacterial growth and incubated at 37°C for a couple of days. The pH values of both filtrates were measured to ~5. Half of the filtrates were also freeze dried to achieve higher concentration.

Cultures of *Bacillus amyloliquefaciens*, strains UCMB 5036, 5033 and 5113 were filtrated (0.2 μm Millipore filter) and then incubated to check for bacterial growth before use.

Screening for antibacterial effect

Sterile paper circles saturated with diluted (0, 1:1, 1:10, 1:100, 1:1000, 1:10 000) filtrates of marine biological waste products, negative control (MQ H_2O) and two positive controls (Spectinomycin 10 mg/mL and Kanamycin 50 mg/mL) were placed on nutrient plates containing *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas* or *Staphylococcus aureus.* The plates were then incubated at 37°C for up to a week. These four bacteria were also screened on LB plates against unfiltered marine waste products, heat inactivated unfiltrated marine biological waste products, filtrate of *B. amyloliquefaciens* strain 5036 and honey and on BHI plates against concentrated filtrate of marine biological waste products and filtrates of *B. amyloliquefaciens* strains 5033 and 5113 at 37°C.

New strains of *P. aeruginosa*, *E.coli* and *S. aureus* (8325-4) were obtained and tested against unfiltrated and concentrated filtrate of marine biological waste products, filtrates of *B. amyloliquefaciens* strains 5036, 5033 and 5113, Kanamycin and Spectinomycin on BHI plates at 37°C.

Bipolaris sorokiniana on PDA plates were screened against unfiltrated, filtrated and concentrated filtrate of marine biological waste products, *B. amyloliquefaciens* strains 5036, 5033 and 5113, Spectinomycin and Kanamycin at daily rhythm conditions. The effect of unfiltrated biological waste products 1 and 2, Spectinomycin, Kanamycin, *B. amyloliquefaciens* strains 5036 and 5033 on Botrytis growing on PDA plates were also tested under daily rhythm conditions.

Staphylococcus equi grown on blood agar plates were also screened against all of the substances above except filtrated marine biological waste products and honey.

Results

Glucosinolates and myrosinase

Glucose concentration in the glucosinolate extracts

To determine the glucosinolate content of the various extracts after ion exchange chromatography, samples from the flow through and the elution fractions were incubated with myrosinase. Thereafter, a commercial reagent was added that converts the released glucose into a colored product measured at 550 nm.

When measuring the pooled mustard extract for the first time it was diluted to four concentrations (100, 75, 50 and 25 %) for future references plus a blank and the concentrations were incubated for two hours at 37°C with the myrosinase. However, the concentrations were so high in some cases that they exceeded the linear scale (appendix A). The concentrations shown are for the diluted extracts (Fig. 5). The values obtained for the undiluted and the 75 % extracts are not shown in the diagram because the glucose amount in the blank exceeded the test samples. As can be seen, the flow through fraction contains more glucose than the elution fraction.

The same procedure was followed for the cabbage extract. However, the results were inconclusive since the readings exceeded the scale to up the double the amount so no diagram was made (appendix B).

A concentration time line was made for both the mustard and cabbage extract where a different concentration was used for each fraction. The incubation times with myrosinase were 2, 4 and 65 h. Unfortunately, these glucose concentrations also exceeded the scale limit (appendix C and D). Therefore a new concentration time line was made where all fractions for both extracts were kept at 10 % and shorter incubation times were used; 0.5, 1, 2, 4 and 24 h . Again the glucose content was higher in the flow through fraction than in the elution fraction in the mustard extract. An increase in glucose concentration could be seen in the elution fraction at 1 – 2 h, which then decreased again at 3 h and forward (Fig. 6, appendix E).

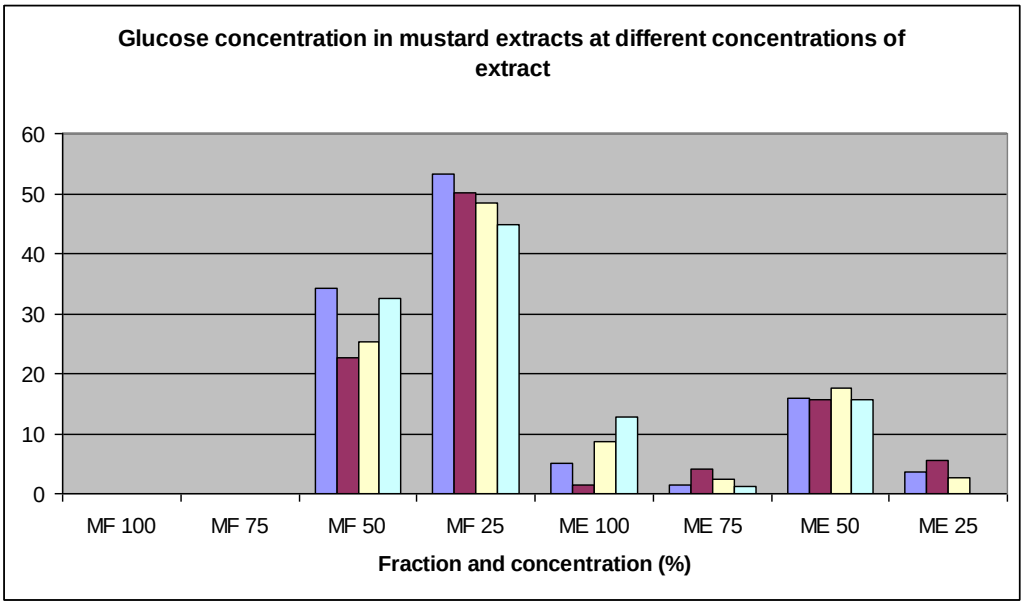

Fig. 5 Glucose concentration in pooled mustard extract. MF and ME denotes flow through fraction and elution fraction respectively. The number means the extract concentration in percent. The glucose concentration is that of the diluted extract.

When a fraction concentration of 10 % was used, some measurable results could be obtained from the analysis of the cabbage extract, but only from the flow through fraction. However, the results were inconclusive due to large differences between the samples (Fig. 7). The incubation times and samples missing is due to the blank that apparently had a higher glucose concentration than the samples. The elution fraction once again exceeded the scale (appendix F).

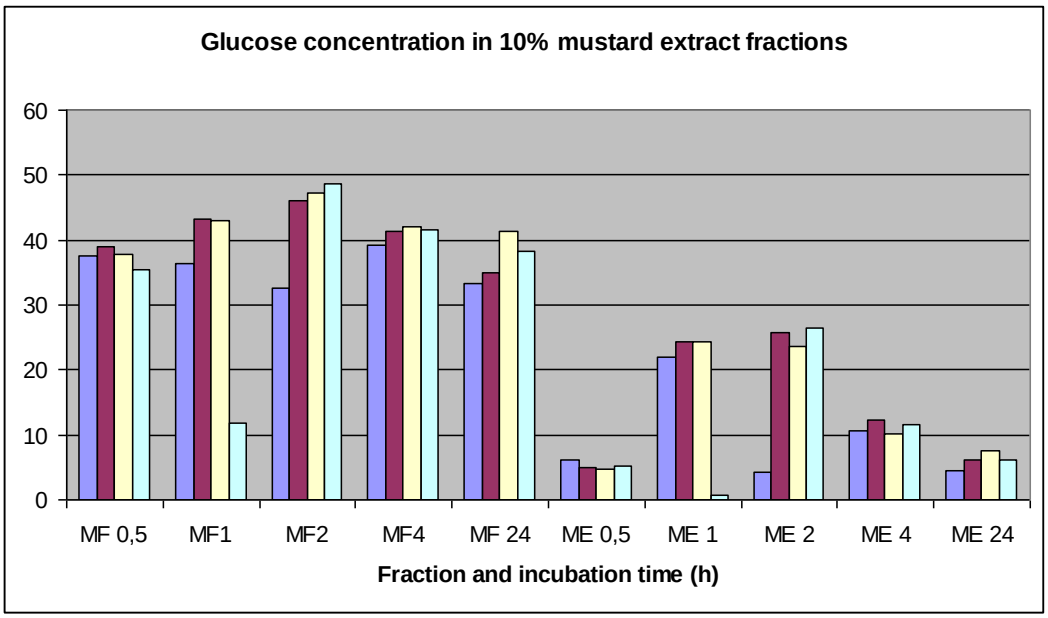

Fig. 6 Concentration time line for pooled mustard extract with a fraction concentration of 10%. MF and ME denotes flow through fraction and elution fraction respectively. The number represents the incubation time (h)

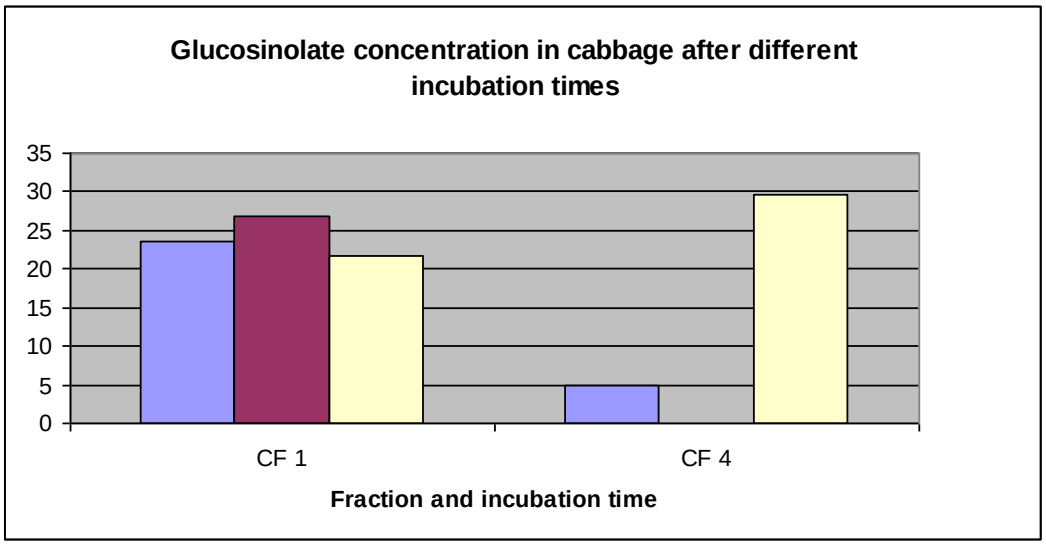

Fig. 7 10% concentration time line for cabbage extract. CF represents flow through fraction and the numbers correspond to the incubation time (h).

A concentration time line was also made for mustard extract three with 10% fraction concentration and incubations times of 0.5, 1, 2 and 4 h (Fig. 8). While some glucose was detected in the flow through fraction it was only a tenth of the glucose amount found in pooled extract one and two and no glucose was measured in the elution fraction (appendix G).

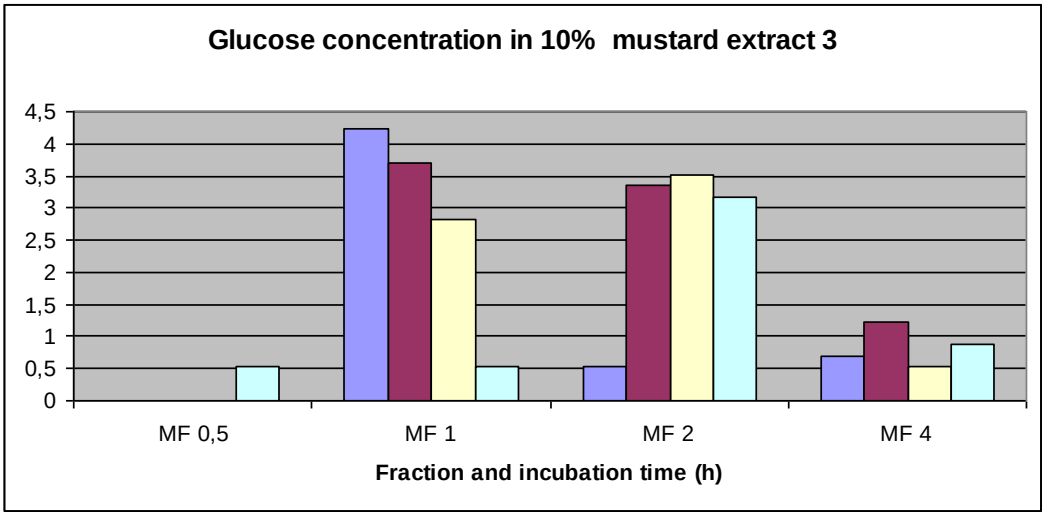

Fig. 8. Concentration time line for mustard extract three with a fraction concentration of 10%. MF denotes flow through fraction and the number stands for incubation time (h)

The concentration time line for the flow through fraction from extract four at 10 % concentration almost reached the same glucose level as pooled extract one and two, but only between 1 – 2 h of incubation, otherwise it was very low. The glucose content in the elution fraction was not anywhere near the value of the pooled extract (Fig. 9, appendix H).

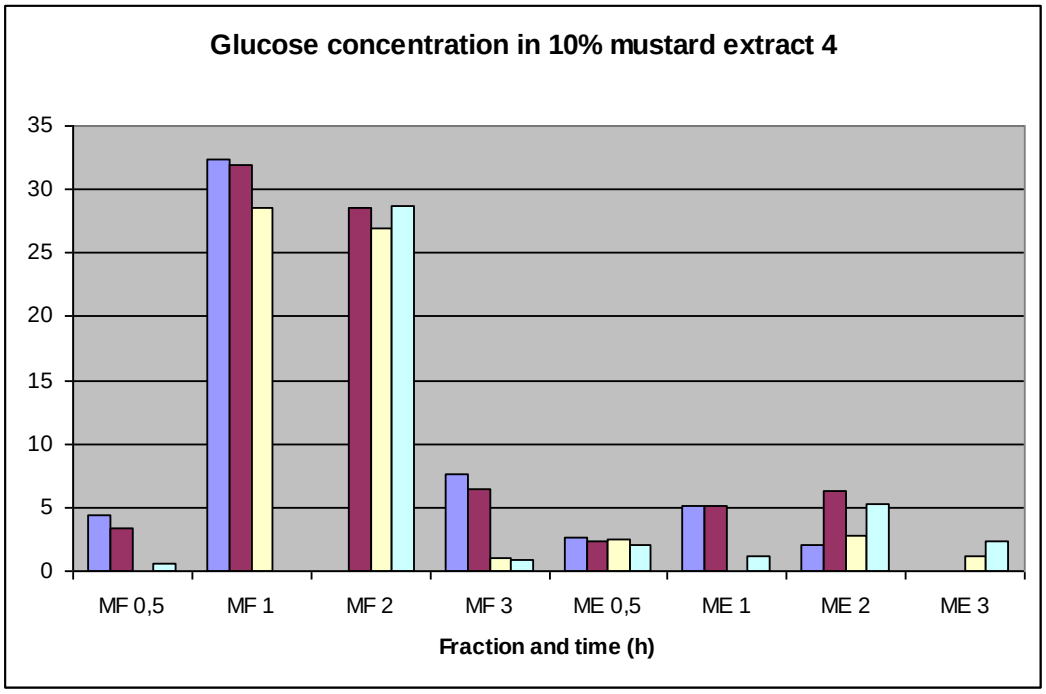

Fig. 9 Concentration time line for mustard extract four with a fraction concentration of 10%. MF represents flow through fraction and the number stands for incubation time (h).

Screening for glucosinolate breakdown

L. maculans spores were spread on medium plates containing barium and different glucosinolate substrates to screen for potential myrosinase activity. *L. maculans* was positive for sinigrin degradation, which was revealed by the opaque barium sulfate area around the fungi as previously described by Sakorn et al. (2002).

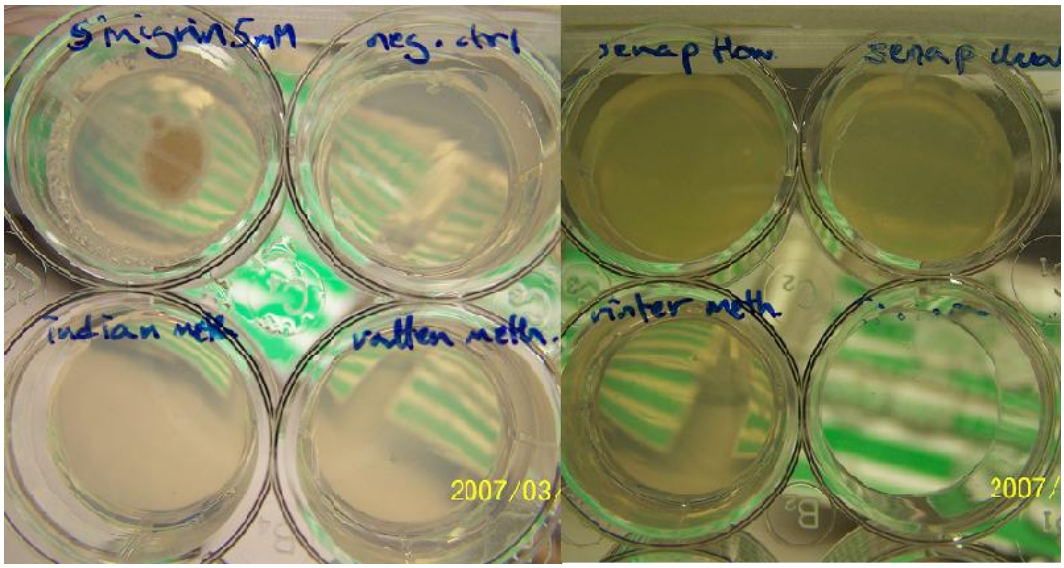

Fig. 10 Screening for glucosinolate degradation in *L. maculans*. The substrates are from top left to bottom right: Sinigrin, neg. control, mustard extract flow through fraction, mustard extract elution fraction, Indian cress, Water cress and Winter cress.

However, no growth was observed on the plates containing other glucosinolate substrates nor on the negative control (Fig. 10).

Botrytis was also screened against the same glucosinolate extracts as *L. maculans*. However Botrytis grew on all plates including the negative control (blank) albeit it can be difficult to see in the picture (Fig. 11).

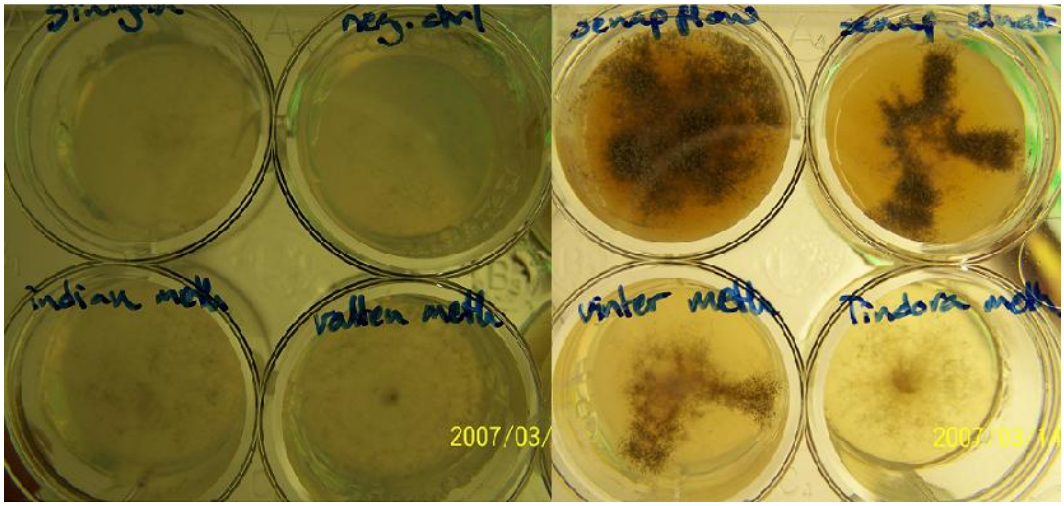

Fig. 11 Screening for myrosinase activity in Botrytis. The substrates are from top left to bottom right: Sinigrin, neg. control, mustard extract flow through fraction, mustard extract elution fraction, Indian cress, Water cress, Winter cress and Tindora.

The *L. agilis* strain R16 that previously had been shown to have a degradative effect on sinigrin, was also screened, but on MRS plates lacking sulfate and glucose but containing either barium or sinigrin and barium. The results were that the bacteria grew on all three substrates (pictures not shown).

All the other bacteria were similarly tested for myrosinase activity. However only three bacteria (*L. johnsonii*, *L. plantarum* and *L.* "new species 2 rat") showed some signs of potential degradation of sinigrin (Fig. 12).

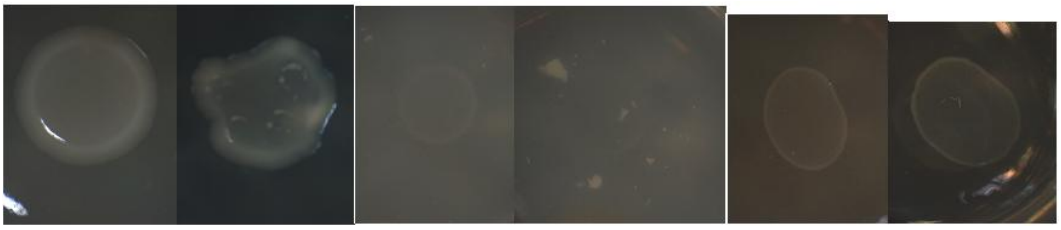

Fig. 12 Results of screening for myrosinase activity.
From left to right: *L. plantarum* (5mM sinigrin), *L. plantarum* (neg. ctrl with barium), *L. johnsonii* (0,25mM sinigrin), *L. johnsonii* (neg. ctrl with barium), *L.* "new species 2 rat" (5mM sinigrin) and *L.*" new species 2 rat" (neg ctrl with out barium).

These three species were further tested by incubation in MRS-G containing either sinigrin or mustard flow through fraction from extract four. Samples were taken at different time points and the glucose content was measured using spectrophotometry. A higher amount of glucose was found when the bacteria were induced by sinigrin than when the flow through fraction was used as can be seen in tables 1 and 2.

Table 1. Glucose content in medium after induction with 1 mM of sinigrin. B represents blank (negative control).

Sample	Mean abs.	Mean abs - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Mean abs - B abs.	Glucose nmol	Sample - B (nmol)
Neg. Ctrl	0.134		0		Neg. Ctrl	0.134		0	
Stand. 1	0.176		5.55		Stand. 1	0.176		5.55	
Stand. 2	0.21		11.1		Stand. 2	0.21		11.1	
Stand. 3	0.277		16.65		Stand. 3	0.277		16.65	
Stand. 5	0.335		27.75		Stand. 5	0.335		27.75	
Stand. 10	0.521		55.5		Stand. 10	0.521		55.5	
Stand. 15	0.7		83.25		Stand. 15	0.7		83.25	
Stand. 20	0.949		111		Stand. 20	0.949		111	
John. 2	0.198	0.05	8.533	7.044	R16 2	0.076	-	-8.655	-
John. 2 B	0.148		1.489		R16 2 B	0.075		-8.796	
John. 6	0.133	-	-0.624	-	R16 6	0.147	-	1.348	-
John. 6 B	0.138		0.08		R16 6 B	0.267		18.255	
John. 24	0.825	-	96.871	-	R16 24	1.17	1.027	145.479	144.695
John. 24 B	0.084		-7.528		R16 24 B	0.143		0.784	
John. 48	0.924	0.171	110.82	24.093	R16 48	1.83	1.561	238.466	219.929
John. 48 B	0.753		86.727		R16 48 B	0.269		18.537	
Plant. 2	0.129	-	-1.188	-	New spec (2) 2	0.618	0.073	67.707	10.285
Plant. 2 B	0.113		-3.442		New spe(2) 2 B	0.545		57.422	
Plant. 6	0.654	0.118	72.779	16.625	New spec (2) 6	0.497	0.047	50.66	6.622
Plant. 6 B	0.536		56.154		New spe(2) 6 B	0.45		44.038	
Plant. 24	0.708	0.062	80.387	8.735	New spec(2) 24	0.085	-	-7.387	-
Plant. 24 B	0.646		71.652		New sp(2) 24 B	0.058		-11.191	
Plant. 48	1.15	0.099	142.661	13.948	New spec(2) 48	0.304	0.233	23.468	-
Plant. 48 B	1.051		128.713		New sp(2) 48 B	0.071	-	-9.36	

Table 2. Glucose content in medium after induction with mustard extract (4) flow through fraction.

Sample	Mean abs.	Mean abs - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Mean abs - B abs.	Glucose (nmol)	Sample. - B (nmol)
Neg. Ctrl	0.269				Neg. Ctrl	0.269			
Stand. 1	0.288		5.55		Stand. 1	0.288		5.55	
Stand. 2	0.307		11.1		Stand. 2	0.307		11.1	
Stand. 3	0.3337		16.65		Stand. 3	0.337		16.65	
Stand. 5	0.419		27.75		Stand. 5	0.419		27.75	
Stand. 10	0.597		55.5		Stand. 10	0.597		55.5	
Stand. 15	0.816		83.25		Stand. 15	0.816		83.25	
Stand. 20	1.062		111		Stand. 20	1.062		111	
John. 2	0.739	0.157	70.663	21.428	R16 2	0.349	0.126	17.435	17.197
John. 2 B	0.582		49.235		R16 2 B	0.223		0.238	
John. 6	0.365	-	19.619	-	R16 6	0.108		-15.457	-
John. 6 B	0.15		-9.725		R16 6 B	0.161		-8.224	
John. 24	0.163	-	-7.951	-	R16 24	1.897	-	228.71	-
John. 24 B	0.154		-9.179		R16 24 B	2.507		311.964	
Plant. 2	0.592	0.112	50.6	15.286	New spec (2) 2	0.614	0.112	53.603	15.286
Plant. 2 B	0.48		35.314		New spe(2) 2 B	0.502		38.317	
Plant. 6	0.739	-	70.663	-	New spec (2) 6	0.463	-	32.994	-
Plant. 6 B	1.932		233.487		New spe(2) 6 B	0.544		44.049	
Plant. 24	1.998	-	242.495	-	New spec(2) 24	1.803	-	215.88	-
Plant. 24 B	2.449		304.048		New sp(2) 24 B	2.261		278.389	

The bacterial pellets from the samples taken at 6 and 24 h from the mustard induction were rehydrated and diluted to four concentrations (100, 10, 1 and 0.1 %) which were spread onto MRS plates to make a viable count analysis. The results (Table 3) show that most of the bacteria survived in the negative control.

Table 3. Viable count of the bacteria that were induced by mustard extract. The number in the parenthesis represents the time point when they were collected, 2 = 6 h and 3 = 24 h. The percentage is their concentration.

Sample (positive)	VC	Sample (Blanks)	VC	Sample (pos.)	VC	Sample (pos.)	VC	Sample (B)	VC	Sample (pos.)	VC	Sample (B)	VC
John. (2) 1%	~700			Plant. (2) 0.1 %	~1904	R16 (2) 0.1 %	~2032	R16 (2) 0.1 %	30	New specie 2 (2) 1 %	31	New specie 2 (2) 1 %	16
		John. (3) 100 %	~1312					R16 (2) 1 %	~310	New specie 2 (2) 10 %	~459	New specie 2 (2) 10 %	~340
												New specie 2 (2) 100 %	~1200
												New specie 2 (3) 1 %	~215
										New specie 2 (3) 10 %	~956	New specie 2 (3) 10 %	~1024

Antibacterial effects of marine biological waste products from food processing

Screening for antibacterial effect

Marine biological waste products from food processing along with honey and three strains of *B. amyloliquefaciens* (5033, 5036 and 5113) were tested for antimicrobial activity against four bacteria (*P. aeruginosa* (two strains), *S. aureus* (two strains), *E.coli* (two strains) and *S. epidermidis* (one strain), and two fungi,

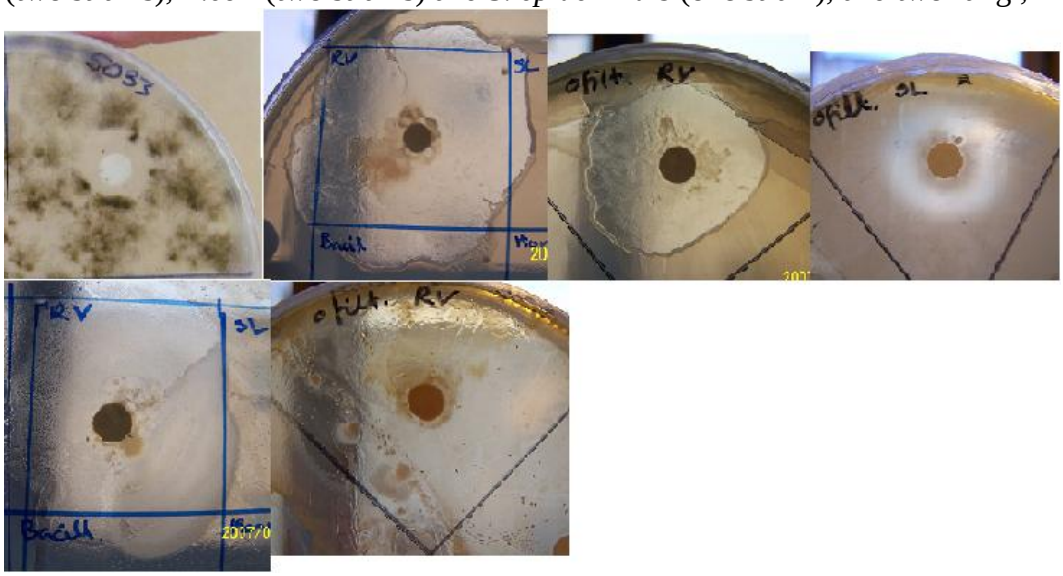

Fig. 13 Antimicrobial effect of various substances/microorganisms.
The panel show from left to right: the effect of *B. amyloliquefaciens* strain 5033 against *Bipolaris sorokiniana*, effect of untreated biological waste product 1 upon two strains of *E. coli*, effect of untreated biological waste product 2 on *P. aeruginosa*, effect of untreated biological waste product 1 against both *S. aureus*.

Bipolaris sorokiniana and Botrytis. Kanamycin and Spectinomycin were used as positive controls. Honey had no effect on any microorganism. Of the substances only *B. amyloliquefaciens* strain 5033 had a slight inhibitory effect on Bipolaris. None of the substances had a deterrent effect against Botrytis. Marine biological waste product 1 that had not been treated in any way had a lytic effect on both strains of *E.coli*. It also had some kind of effect against *S. aureus*. Marine biological waste product 2 was the only one that had an inhibitory effect against *P. aeruginosa* (Fig. 13).

Although not shown here, both marine biological waste products 1 and 2 contained some kind of microbes that could grow and compete with the bacteria growing on the plates, while not killing the bacteria.

Discussion

Glucosinolates and myrosinase

Glucosinolates are organic compounds in certain plants whose metabolites produced via the degradation by myrosinase have been shown to counteract tumor cells in both humans and animals. However, most humans eat their vegetables cooked or processed in another way, which is a problem since heating inactivates the myrosinase naturally present in the plants. Accordingly, microorganisms containing myrosinase might be supplemented to the diet to enhance the degradation of glucosinolates in the gut.

To investigate this possibility several bacteria that have previously been reported to express myrosinase activity as well as related bacteria were first tested for glucosinolate breakdown via a barium sulfate test. The results were however inconclusive since it was almost impossible to determine if there had been precipitation due to difficulty in distinguishing between bacterial growth and a possible precipitate. It might be that testing for myrosinase activity this way is not possible for bacteria or that the medium was not opaque enough.

Three bacterial species (*L. plantarum, L. Johnsonii and L.* "new species 2 rat") were nonetheless thought to be positive and were further indirectly tested for myrosinase activity via measurement of glucose content after incubation of bacteria and glucosinolates since one of the breakdown products is glucose. More positive results were obtained when the bacteria were incubated with pure sinigrin than with mustard extract however the results were so sketchy that it was not possible to deduce whether any glucosinolate degradation existed or if the bacteria themselves produced glucose via some alternative metabolic pathway.

Two fungal strains (*L. maculans* and Botrytis) were also tested for glucosinolate degradation. *L. maculans* only grew on sinigrin media and showed obvious precipitation and not on the negative control nor on the other wells containing glucosinolate extracts from mustard and various cresses. The negative results for the mustard and cress extracts can be either because the extracts did not contain enough glucosinolates or that the extracts contained something that inhibited the growth of *L. maculans*. This test should be repeated to see if the same results would be obtained. Botrytis did not seem to distinguish between any of the samples; it grew on all of them including the negative control. However no precipitation could be observed so it might be possible that Botrytis can survive via some other metabolic pathway that does not include formation of sulfate.

Before the bacteria and fungi were grown on the glucosinolate extracts both cabbage and mustard extracts were incubated with purified myrosinase before detection for glucose to see if they contained glucosinolates. When the mustard extracts were tested the flow through fractions contained more glucose than elution fraction which was expected since they should have contained naturally occurring glucose, however, the amounts in the elution fractions differed between mustard batches and even within the same batch when sampled at different times. The differences in the elution fractions between the extracts could be due to different handling during the extraction process. Nonetheless the changes were so minor that they should not have had such an impact upon the glucosinolate concentration. Another odd observation was that the glucose content in the elution fraction first rose during incubation and peaked at 1 – 2 hours of incubation which was expected, yet after this time period the glucose content decreased which was

not anticipated since there should not have been anything present in the extract that would degrade the glucose. Even if the glucose content did not rise after 2 h it should at least have remained constant for the whole duration. An explanation for this anomaly could not be deduced. It is possible that these variations were due to faulty processing or human error during the measurement.

To see if the odd results were due to human error or an uncontrolled and unknown variable these tests should be redone and the procedures modified.

Antibacterial effects of marine biological waste products from food processing

Multi-resistant bacteria are a growing global problem and has stimulated intensive efforts to find novel antibacterial substances in unlikely and unexplored places e.g. from sea living creatures. Field studies had been conducted where it had been found that marine biological waste products from food processing had a small inhibitory effect upon occurrence of fungi on cereals.

These substances were tested under in vitro conditions to study if they possibly could have an antibacterial effect on human pathogenic bacteria. When the bacteria were grown in the presence of the substances it was shown that they both had an adverse effect on the bacteria. However, this effect was probably due to a biological effect - i.e. some biotic agent(s) was killing/neutralizing the bacteria – rather than a chemical effect. This was proven in that the unprocessed substances cleared the plates of the bacteria whilst nurturing some other organism naturally occurring in the substance, but they had no effect whatsoever when they were filtrated before application to the bacteria.

These substances were also tested unprocessed as well as filtrated on two fungal species (*B. sorokiniana* and Botrytis), but no effect could be observed. This lack of effect could be due to that they were only tested on fully grown specimen; if they had been tested on spores the results might have been different. While not having any effect upon fully formed fungi they might have prevented the spores from germinating.

Since positive results were obtained this research venue should be pursued. A way to identify whether the inhibition is due to a chemical or biological agent would be to incubate the marine sample with a small amount of desired bacteria in a liquid culture. The culture would then be purified and filtrated to remove cells and assayed using a diffusion assay to see if the inhibition existed in the filtrate. This would enable the microorganisms in the marine sample to react to the bacteria before being assayed and thus be able to produce, if possible, an agent against the bacteria. This might explain why the filtrated samples did not have an effect upon the bacteria while the untreated ones did have one.

More diffusion screenings should be performed and attempts to isolate the active substance should be made. The isolation could be achieved by separating the marine sample using thin-layer chromatography, coating the silica plate in agar and growing the wanted bacteria on top of the silica plate to observe if any inhibition occurs on a specific band. If inhibition occurs that band can be analyzed using high performance liquid chromatography and mass spectrometry to identify the components in the band. The components should then be individually screened against the bacteria to see which is active against the bacteria. If an active component is found it should then be screened against known antibacterial substances to see if it is an already known compound. If the component is

unknown, further testing should be performed to analyze its origin, original function and its effect and pathway upon the human pathogenic bacteria.

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Appendix A

Glucose content in flow through fraction (MF) and elution fraction (ME) in the first mustard extract. The numbers are the extract concentration in % and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl	0		0		Neg. Ctrl	0		0	
Stand. 2	0.119		11.1		Stand. 2	0.122		11.1	
Stand. 3	0.159		16.65		Stand. 3	0.175		16.65	
Stand. 5	0.231		27.75		Stand. 5	0.233		27.75	
Stand. 10	0.388		55.5		Stand. 10	0.397		55.5	
Stand. 15	0.785		83.25		Stand. 15	0.544		83.25	
Stand. 20	0.757		111		Stand. 20	0.761		111	
MF 100	0.46	-	58.331	-	ME 100	0.514	0.031	73.992	5.046
MF 100	0.44	-	55.544	-	ME 100	0.492	0.009	70.411	1.465
MF 100	0.402	-	50.248	-	ME 100	0.537	0.054	77.737	8.791
MF 100	0.401	-	50.108	-	ME 100	0.561	0.078	81.644	12.698
MF B100	0.805		106.413		ME B100	0.483		68.946	
MF 75	0.412	-	51.642	-	ME 75	0.561	0.009	81.664	1.485
MF 75	0.46	-	58.331	-	ME 75	0.577	0.025	84.248	4.069
MF 75	0.494	-	63.07	-	ME 75	0.567	0.015	82.621	2.442
MF 75	0.53	-	68.087	-	ME 75	0.56	0.008	81.481	1.302
MF B75	0.693		90.804		ME B75	0.552		80.179	
MF 50	0.777	0.246	102.51	34.284	ME 50	0.627	0.097	92.388	15.791
MF 50	0.694	0.163	90.943	22.717	ME 50	0.626	0.096	92.226	15.629
MF 50	0.713	0.182	93.591	25.365	ME 50	0.638	0.108	94.179	17.582
MF 50	0.764	0.233	100.699	32.473	ME 50	0.626	0.096	92.226	15.629
MF B50	0.531		68.226		ME B50	0.53		76.597	
MF 25	0.783	0.382	103.347	53.239	ME 25	0.387	0.022	53.317	3.582
MF 25	0.761	0.36	100.281	50.173	ME 25	0.399	0.034	55.271	5.536
MF 25	0.748	0.347	98.469	48.361	ME 25	0.381	0.016	52.34	2.605
MF 25	0.723	0.322	94.985	44.877	ME2	0.354	-	47.945	-
MF B25	0.401		50.108		ME B25	0.365		49.735	

Appendix B

Glucose content in flow through fraction (CF) and elution fraction (CE) in the first cabbage extract. The numbers are the extract concentration in % and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample. - B (nmol)
Neg. Ctrl	0		0		Neg. Ctrl	0		0	
Stand. 2	0.119		11.1		Stand. 2	0.122		11.1	
Stand. 3	0.159		16.65		Stand. 3	0.175		16.65	
Stand. 5	0.231		27.75		Stand. 5	0.233		27.75	
Stand. 10	0.388		55.5		Stand. 10	0.397		55.5	
Stand. 15	0.785		83.25		Stand. 15	0.544		83.25	
Stand. 20	0.757		111		Stand. 20	0.761		111	
CF 100	1.29	-	174.006	-	CE 100	1.782	-	280.419	-
CF 100	1.501	-	203.412	-	CE 100	1.692	-	265.768	-
CF 100	1.972	-	269.054	-	CE 100	1.7	-	267.070	-
CF 100	2.033	-	277.555	-	CE 100	1.768	-	278.140	-
CF B100	1.996		272.399		CE B100	1.816		285.955	
CF 75	1.834	-	249.821	-	CE 75	1.754	-	275.861	-
CF 75	1.629	-	221.251	-	CE 75	1.722	-	270.652	-
CF 75	1.809	-	246.337	-	CE 75	1.926	-	303.862	-
CF 75	1.696	-	230.589	-	CE 75	1.715	-	269.512	-
CF B 75	1.274		171.776		CE B75	1.62		254.046	
CF 50	0.871	-	115.611	-	CE 50	1.615	-	253.232	-
CF 50	0.798	-	105.437	-	CE 50	1.666	-	261.535	-
CF 50	0.876	-	116.308	-	CE 50	1.754	-	275.861	-
CF 50	0.741	-	97.493	-	CE 50	1.624	-	254.697	-
CF B50	0.987		131.778		CE B50	1.745		274.396	
CF 25	0.568	-	73.383	-	CE 25	2.018	-	318.840	-
CF 25	0.677	-	88.574	-	CE 25	2.088	-	330.235	-
CF 25	0.838	-	111.012	-	CE 25	2.113	-	334.305	-
CF 25	0.824	-	109.061	-	CE 25	2.149	-	340.166	-
CF B25	1.048		140.279		CE B25	2.128		336.747	

Appendix C

Glucose content in flow through fraction (MF) and elution fraction (ME) in the first concentration time line for mustard. The concentrations used were 25% for flow through and 50% for elute. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample. - B (nmol)
Neg. Ctrl					Neg. Ctrl				
Stand. 2	0.134		11.10		Stand. 2	0.142		11.10	
Stand. 3	0.162		16.65		Stand. 3	0.180		16.65	
Stand. 5	0.263		27.75		Stand. 5	0.250		27.75	
Stand. 10	0.460		55.50		Stand. 10	0.369		55.50	
Stand. 15	0.645		83,25		Stand. 15	0.456		83.25	
Stand. 20	0.793		111		Stand. 20	0.799		111	
MF 2	1.077	-	149.918	-	ME 2	1.505	-	244.417	-
MF 2	1.151	-	160.898	-	ME 2	1.377	-	222.667	-
MF 2	1.115	-	155.556	-	ME 2	1.325	-	213.831	-
MF 2	1.124	-	156.891	-	ME 2	1.226	-	197.008	-
MF 2B	0.909		124.99		ME 2B	0.951		150.280	
MF 4	1.086	-	151.253	-	ME 4	1.237	-	198.878	-
MF 4	1.089	-	151.698	-	ME 4	1.156	-	185.114	-
MF 4	1.012	-	140.273	-	ME 4	1.301	-	209.753	-
MF 4	0.989	-	136.860	-	ME 4	0.934	-	147.391	-
MF 4B	0.857		117.274		ME 4B	1.214		194.969	
MF 65	1.095	-	152.588	-	ME 65	1.135	-	181.545	-
MF 65	1.162	-	162.530	-	ME 65	1.151	-	184.264	-
MF 65	1.134	-	158.375	-	ME 65	1.133	-	181.206	-
MF 65	1.157	-	161.788	-	ME 65	1.095	-	174.749	-
MF 65B	0.942		129.886		ME 65B	0.991		157.077	

Appendix D

Glucose content in flow through fraction (CF) and elution fraction (CE) in the first concentration time line for cabbage. The concentrations used were 75% for flow through and 75% for elute. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl					Neg. Ctrl				
Stand. 2	0.134		11.10		Stand. 2	0.142		11.10	
Stand. 3	0.162		16.65		Stand. 3	0.180		16.65	
Stand. 5	0.263		27.75		Stand. 5	0.250		27.75	
Stand. 10	0.460		55.50		Stand. 10	0.369		55.50	
Stand. 15	0.645		83.25		Stand. 15	0.456		83.25	
Stand. 20	0.793		111		Stand. 20	0.799		111	
CF 2	2.773	-	401.567	-	CE 2	1.765	-	288.597	-
CF 2	2.800	-	405.573	-	CE 2	1.418	-	229.633	-
CF 2	2.948	-	427.533	-	CE 2	1.417	-	229.464	-
CF 2	2.486	-	358.983	-	CE 2	1.375	-	222.327	-
CF 2B	2.054		294.883		CE 2B	1.353		218.589	
CF 4	2.115	-	303.934	-	CE 4	1.328	-	214.340	-
CF 4	1.970	-	282.419	-	CE 4	1.398	-	226.235	-
CF 4	2.220	-	319.514	-	CE 4	1.782	-	291.485	-
CF 4	2.414	-	348.299	-	CE 4	1.476	-	239.489	-
CF 4B	2.875		416.702		CE 4B	1.467		237.960	
CF 65	2.255	-	324.707	-	CE 65	1.286	-	207.204	-
CF 65	2.125	-	305.418	-	CE 65	1.492	-	242.208	-
CF 65	2.439	-	352.009	-	CE 65	1.669	-	272.284	-
CF 65	2.439	-	352.009	-	CE 65	1.398	-	226.235	-
CF 65B	1.598		227.233		CE 65	1.256		202.106	

Appendix E

Glucose content in flow through fraction (MF) and elution fraction (ME) in the second concentration time line for mustard where the concentration was 10%. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl	0		0		Neg. Ctrl	0		0	
Stand. 2	0.113		11.10		Stand. 2	0.142		11.10	
Stand. 3	0.158		16.65		Stand. 3	0.189		16.65	
Stand. 5	0.249		27.75		Stand. 5	0.257		27.75	
Stand. 10	0.444		55.50		Stand. 10	0.452		55.50	
Stand. 15	0.622		83.25		Stand. 15	0.660		83.25	
Stand. 20	0.811		111		Stand. 20	0.844		111	
MF 0.5	0.577	0.261	76.469	37.634	ME 0.5	0.281	0.044	30.568	6.248
MF 0.5	0.587	0.271	77.911	39.076	ME 0.5	0.272	0.035	29.290	4.970
MF 0.5	0.578	0.262	76.613	37.778	ME 0.5	0.271	0.034	29.148	4.828
MF 0.5	0.562	0.246	74.306	35.471	ME 0.5	0.274	0.037	29.574	5.254
MF 0.5B	0.316		38.835		ME 0.5B	0.237		24.320	
MF 1	0.571	0.253	75.604	36.481	ME 1	0.380	0.154	44.627	21.869
MF 1	0.618	0.300	82.381	43.258	ME 1	0.397	0.171	47.041	24.283
MF 1	0.616	0.298	82.093	42.970	ME 1	0.398	0.172	47.183	24.425
MF 1	0.400	0.082	50.947	11.824	ME 1	0.263	0.037	28.012	0.710
MF 1B	0.318		39.123		ME 1B	0.226		22.758	
MF 2	0.507	0.226	66.376	32.588	ME 2	0.258	0.030	27.302	4.260
MF 2	0.601	0.320	79.930	46.142	ME 2	0.409	0.181	48.745	25.703
MF 2	0.608	0.092	80.939	47.151	ME 2	0.395	0.167	46.757	23.715
MF 2	0.618	0.337	82.381	48.593	ME 2	0.414	0.186	49.455	26.413
MF 2B	0.281		33.788		ME 2B	0.228		23.042	
MF 4	0.516	0.272	67.674	39.221	ME 4	0.252	0.075	26.450	10.650
MF 4	0.530	0.286	69.692	41.239	ME 4	0.263	0.086	28.012	12.212
MF 4	0.536	0.292	70.557	42.104	ME 4	0.249	0.072	26.024	10.224
MF 4	0.532	0.288	69.981	41.528	ME 4	0.259	0.082	27.444	11.644
MF 4B	0.244		28.453		ME 4B	0.177		15.800	
MF 24	0.470	0.231	61.041	33.309	ME 24	0.228	0.031	23.042	4.402
MF 24	0.481	0.242	62.627	34.895	ME 24	0.240	0.043	24.746	6.106
MF 24	0.526	0.287	69.115	41.383	ME 24	0.251	0.054	26.308	7.668
MF 24	0.505	0.266	66.087	38.355	ME 24	0.240	0.043	24.746	6.106
MF 24B	0.239		27.732		ME 24B	0.197		18.640	

Appendix F

Amount of Glucose in flow through fraction (CF) and elution fraction (CE) in the second concentration time line for cabbage where the concentration was 10%. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl	0		0		Neg. Ctrl	0		0	
Stand. 2	0.113		11.10		Stand. 2	0.142		11.10	
Stand. 3	0.158		16.65		Stand. 3	0.189		16.65	
Stand. 5	0.249		27.75		Stand. 5	0.257		27.75	
Stand. 10	0.444		55.50		Stand. 10	0.452		55.50	
Stand. 15	0.622		83.25		Stand. 15	0.660		83.25	
Stand. 20	0.811		111		Stand. 20	0.844		111	
CF 0.5	1.213	-	168.176	-	CE 0.5	2.817	-	390.694	-
CF 0.5	1.220	-	169.186	-	CE 0.5	2.338	-	322.673	-
CF 0.5	1.409	-	196.438	-	CE 0.5	2.509	-	346.956	-
CF 0.5	1.264	-	175.530	-	CE 0.5	2.605	-	360.588	-
CF 0.5B	1.313	-	182.596	-	CE 0.5B	2.523	-	348.944	-
CF 1	0.944	0.164	129.388	23.647	CE 1	2.509	-	346.956	-
CF 1	0.966	0.186	132.560	26.819	CE 1	2.345	-	323.667	-
CF 1	0.731	-	98.675	-	CE 1	2.433	-	336.164	-
CF 1	0.931	0.151	127.514	21.773	CE 1	2.511	-	347.240	-
CF 1B	0.780	-	105.741	-	CE 1B	2.467	-	340.992	-
CF 2	0.821	-	111.652	-	CE 2	2.588	-	358.174	-
CF 2	0.887	-	121.169	-	CE 2	2.703	-	374.505	-
CF 2	0.913	-	124.918	-	CE 2	2.526	-	349.370	-
CF 2	0.722	-	97.377	-	CE 2	2.458	-	339.714	-
CF 2B	1.176	-	162.841	-	CE 2B	2.260	-	311.597	-
CF 4	0.956	0.034	131.119	4.903	CE 4	2.204	-	303.644	-
CF 4	0.900	-	123.044	-	CE 4	2.423	-	334.743	-
CF 4	0.893	-	122.034	-	CE 4	2.323	-	320.543	-
CF 4	1.127	0.205	155.776	29.560	CE 4	2.359	-	325.655	-
CF 4B	0.922		126.216		CE 4B	2.296	-	316.709	-
CF 24	0.779	-	105.596	-	CE 24	2.461	-	340.140	-
CF 24	0.747	-	100.982	-	CE 24	1.907	-	261.469	-
CF 24	0.788	-	106.894	-	CE 24	2.138	-	294.272	-
CF 24	0.745	-	100.694	-	CE 24	2.160	-	297.396	-
CF 24B	0.907	-	124.053	-	CE 24B	2.259	-	311.455	-

Appendix G

Glucose content in flow through fraction (MF) and elution fraction (ME) in 10% concentration time line for mustard extract three. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts.

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample – B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl			0		Neg. Ctrl			0	
Stand. 2	0.132		11.10		Stand. 2	0.138		11.10	
Stand. 3	0.168		16.65		Stand. 3	0.183		16.65	
Stand. 5	0.262		27.75		Stand. 5	0.368		27.75	
Stand. 10	0.456		55.50		Stand. 10	0.406		55.50	
Stand. 15	0.642		83.25		Stand. 15	0.634		83,25	
Stand. 20	0.656		111		Stand. 20	0.652		111	
MF 0.5	0.113	-	2.803	-	ME 0.5	0.138	-	0.961	-
MF 0.5	0.110	-	2.275	-	ME 0.5	0.137	-	0.768	-
MF 0.5	0.119	-	3.860	-	ME 0.5	0.135	-	0.382	-
MF 0.5	0.123	0.003	4.564	0.528	ME 0.5	0.134	-	0.189	-
MF 0.5B	0.120	-	4.036		ME 0.5B	0.125	-	-1.546	
MF 1	0.135	0.024	6.677	4.226	ME 1	0.118	-	-2.896	-
MF 1	0.132	0.021	6.149	3.698	ME 1	0.119	-	-2.703	-
MF 1	0.127	0.016	5.268	2.817	ME 1	0.121	-	-2.318	-
MF 1	0.114	0.003	2.979	0.528	ME 1	0.119	-	-2.703	-
MF 1B	0.111		2.451		ME 1B	0.116		-3.282	
MF 2	0.120	0.003	4.036	0.529	ME 2	0.120	-	-2.511	-
MF 2	0.136	0.019	6.853	3.346	ME 2	0.122	-	-2.125	-
MF 2	0.137	0.020	7.029	3.522	ME 2	0.120	-	-2.511	-
MF 2	0.135	0.018	6.677	3.170	ME 2	0.116	-	-3.282	-
MF 2B	0.117		3.507		ME 2B	0.115		-3.475	
MF 4	0.111	0.004	2.451	0.705	ME 4	0.115	-	-3.475	-
MF 4	0.114	0.007	2.979	1.233	ME 4	0.111	-	-4.246	-
MF 4	0.110	0.003	2.275	0.529	ME 4	0.113	-	-3.860	-
MF 4	0.112	0.005	2.627	0.881	ME 4	0.114	-	-3.668	-
MF 4B	0.107		1.746		ME 4B	0.117		-3.089	

Appendix H

Glucose content in flow through fraction (MF) and elution fraction (ME) in 10% concentration time line for mustard extract four. Numbers are the incubation time (h) and B stands for blank. The concentrations are for the diluted extracts

Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)	Sample	Mean abs.	Abs. - B abs.	Glucose (nmol)	Sample - B (nmol)
Neg. Ctrl	0.187		0		Neg. Ctrl	0.184		0	
Stand. 2	0.237		11.10		Stand. 2	0.250		11.10	
Stand. 3	0.295		16.65		Stand. 3	0.287		16.65	
Stand. 5	0.284		27.75		Stand. 5	0.316		27.75	
Stand. 10	0.510		55.50		Stand. 10	0.563		55.50	
Stand. 15	0.736		83.25		Stand. 15	0.719		83.25	
Stand. 20	0.983		111		Stand. 20	1.020		111	
MF 0.5	0.241	0.033	15.123	4.435	ME 0.5	0.238	0.020	12.714	2.652
MF 0.5	0.233	0.025	14.048	3.360	ME 0.5	0.236	0.018	12.449	2.387
MF 0.5	0.208	0	10.688	0	ME 0.5	0.237	0.019	12.582	2.520
MF 0.5	0.212	0.004	11.225	0.537	ME 0.5	0.234	0.016	12.184	2.122
MF 0.5B	0.208		10.688		ME 0.5B	0.218		10.062	
MF 1	0.478	0.241	46.977	32.391	ME 1	0.227	0.039	11.256	5.171
MF 1	0.475	0.238	46.574	31.988	ME 1	0.227	0.039	11.256	5.171
MF 1	0.449	0.212	43.079	28.493	ME 1	0.145	-	0.384	-
MF 1	0.235	-	14.317	-	ME 1	0.197	0.009	7.278	1.193
MF 1B	0.237		14.586		ME 1B	0.188		6.085	
MF 2	0.222	-	12.569	-	ME 2	0.194	0.016	6.881	2.122
MF 2	0.452	0.213	43.483	28.629	ME 2	0.226	0.048	11.123	6.364
MF 2	0.439	0.200	41.735	26.881	ME 2	0.199	0.021	7.543	2.784
MF 2	0.453	0.214	43.617	28.763	ME 2	0.218	0.040	10.062	5.303
MF2B	0.239		14.854		ME 2B	0.178		4.759	
MF 3	0.237	0.057	14.586	7.662	ME 3	0.198	-	7.411	-
MF 3	0.228	0.048	13.376	6.452	ME 3	0.196	-	7.146	-
MF 3	0.188	0.008	8.000	1.076	ME 3	0.226	0.009	11.123	1.193
MF 3	0.187	0.007	7.865	0.941	ME 3	0.235	0.018	12.316	2.386
MF 3B	0.180		6.924		ME 3B	0.217		9.930	

ISSN 1651-5196 Nr 101
Uppsala 2009

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