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3 Optimizing the Use of *Sinapis alba* Seed Meal Extracts as a Source of Thiocyanate (SCN⁻) for the
4 Lactoperoxidase System

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Abstract:

The multi-component Lactoperoxidase system (LPSys) is used for improving food safety and requires thiocyanate (SCN^-) as a substrate for the generation of antimicrobial hypothiocyanite (OSCN^-). Production of OSCN^- is usually limited by intrinsic concentrations of SCN^- or else SCN^- must be supplied from a synthetic source. Our objective was to optimize the production of OSCN^- in the LPSys using SCN^- derived from glucosinolates present in yellow mustard (*Sinapis alba*) seed meal. Thiocyanate was produced from *S. alba* seed meal by shaking a 1:20 (w:w) meal: H_2O mixture at 70 °C for 10 min. Increasing the exogenous H_2O_2 to an equimolar concentration (500 μM) of SCN^- further increased OSCN^- production, whereas H_2O_2 concentrations in excess of SCN^- enhanced OSCN^- decay. Glucose and glucose oxidase (GOx) were substituted for H_2O_2 to sustain OSCN^- production resulting in an optimized LPSys containing 4.92 U/mL lactoperoxidase, 64 mM glucose, 342 U/L GOx, and 500 μM SCN^- . Maximum OSCN^- in the optimized system was 40 μM , a concentration that is predicted to have antimicrobial effects, thus demonstrating the potential of the low value *S. alba* seed meal as source of SCN^- for preparing a generally recognized as safe LPSys.

Keywords: Lactoperoxidase system, *Sinapis alba*, antimicrobial, glucosinolates.

50   **Abbreviations:**

51   LPSys: Lactoperoxidase System

52   LP: Lactoperoxidase Enzyme

53   GOx: Glucose Oxidase

54   GRAS: Generally Recognized as Safe

55   ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

56   Nbs₂: 5, 5'-dithiobis (2-nitrobenzoic acid (Nbs₂))

57   DETAPAC: Diethylenetriaminepentaacetic acid

1. Introduction

The lactoperoxidase system (LPSys) is a naturally occurring antimicrobial multi-component system (Elliot, McLay, Kennedy, & Simmonds, 2004) inherently available in raw milk and human body fluids such as saliva. There are three primary components in the LPSys: lactoperoxidase enzyme (LP), hydrogen peroxide (H_2O_2), and thiocyanate (SCN^-). Hydrogen peroxide is produced by a number of microorganisms such as *Lactobacilli*, *Streptococci*, and *Lactococcus* through oxidation of ascorbic acid or oxidation of glucose by glucose oxidase (GOx) (Jooyandeh, Aberoumand, & Nasehi, 2011). Lactoperoxidase catalyzes oxidation of SCN^- by H_2O_2 to produce hypothiocyanite ($OSCN^-$) (Hughes, 1975), which oxidizes essential sulfhydryl groups in proteins and impairs transport systems, membrane stability, and enzyme metabolism (Elliot, et al., 2004). The reactions inhibit growth of both Gram negative (e.g., *E. coli* O157:H7) and Gram positive bacteria (e.g., *Listeria monocytogenes*), thus making the LPSys potentially useful in improving food safety (Elliot, et al., 2004; El-Shenawy et al., 1990; Marshall & Reiter, 1980).

Although investigators have demonstrated the effect of the LPSys in milk (Boulares et al., 2011; Seifu et al., 2005; Seifu, 2007), application in other food systems is limited. For example, application of the LPSys in beef cubes significantly reduced the number of microorganisms including *L. monocytogenes* after 7 d of incubation at 12 °C (Elliot, et al., 2004). Kennedy and others (2000) showed that LPSys inhibited *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* in ground beef. In other food products, the LPSys inhibited *Salmonella enteritidis* in tomato juice, carrot juice, milk, liquid whole egg, and chicken skin extract (Touch, Hayakawa, Yamada, & Kaneko, 2004). Thiocyanate sourced from *S. alba* seed meal could potentially substitute for KSCN in edible coatings for fish preservation (Jasour, Ehsani, Mehryar, & Naghibi, 2015) and to inactivate pathogens in ready to eat meats (Alba, Bravo, & Medina, 2015). In all the cited LPSys

studies, an existing concentration of SCN^- was used or the ion was added from non-food-grade potassium or sodium thiocyanate (KSCN or NaSCN). Therefore, even though the LPSys might be Generally Recognized as Safe (GRAS), it may not be applied in vitro except in raw milk that inherently contains SCN^- to keep the LPSys active.

Provision of a natural source of food-grade SCN^- would facilitate integration of the LPSys into a broader range of food products. *Sinapis alba* (white or yellow mustard) contains a glucosinolate called sinalbin that constitutes over 90% of the total glucosinolate pool. Enzymatic hydrolysis of this glucosinolate by the enzyme myrosinase ([EC 3.2.1.147](#)) produces an unstable isothiocyanate that non-enzymatically undergoes conversion to SCN^- (Botti, Taylor, & Botting, 1995; Pecháček, Velíšek, & Davídek, 2000), thus supplying substrate for the LPSys that can be substituted for KSCN or NaSCN.

Glucosinolate concentrations are highest in the seed of *S. alba* and correspondingly in the seed meal after oil is removed. Although *S. alba* seed can be crushed to produce an oil feedstock for biodiesel with improved cold-flow properties compared to soybean biodiesel (Issariyakul, Dalai, & Desai, 2011), relatively low economic returns limit *S. alba* oil availability. One way to increase economic viability of biodiesel production from *S. alba* seed is to develop value-added products from the seed meal remaining after oil removal. *Brassicaceae* seed meals are most often used for animal feed, however higher value products are required to increase economic returns and expand oilseed acreage (Morra & Borek, 2010). *S. alba* is also a condiment mustard frequently used in the food industry for its emulsifying properties and as a protein concentrate (Cui & Eskin, 1998). Moreover, this extensive use indicates that sensory effects are unlikely to inhibit additional uses related to its serving as a SCN^- source for the LPSys.

In a recent study (Lee, Noh, & Min, 2012), edible film was developed using defatted *S. alba* meal and its effect on *Listeria monocytogenes* investigated. Antimicrobial effects (beyond that of control films with inactivated myrosinase) were observed both in assays involving disks of the film on *L. monocytogenes*-inoculated agar plates and films coated on inoculated salmon. Although SCN⁻ production in the films was quantified, other components of the LPSys were not included. There are no published studies where SCN⁻ generated from mustard meal has been applied in a lactoperoxidase system in the manner that we propose.

Our objective was to optimize the production of OSCN⁻ in the LPSys using SCN⁻ derived from *S. alba* seed meal. Associated with this objective is the need to 1) develop efficient extraction procedures for SCN⁻ and 2) determine if additional compounds in the seed meal extracts affect OSCN⁻ production rates. By developing methods to utilize SCN⁻ produced in *S. alba* seed meal, multiple benefits result including the provision of a natural source of SCN⁻ for the LPSys, development of a value-added product from *S. alba* seed meal produced after oil is extracted for biodiesel, and increased palatability of the residual seed meal as an animal feed.

2. Materials and Methods

2.1. Mustard meal and chemical reagents

All experiments were carried out using meal remaining after cold pressing *S. alba* “IdaGold” seed using a mechanical seed crusher at the University of Idaho (Moscow, ID) (Borek & Morra, 2005). Mustard meal (*S. alba* with ~ 10% oil content) was used as the source of extract for mustard meal SCN⁻ (MM-SCN⁻). Sodium thiocyanate (KSCN, 98.5%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 5, 5'-dithiobis (2-nitrobenzoic acid (Nbs₂), diethylenetriaminepentaacetic acid (DETAPAC), sodium phosphate monobasic

monohydrate, sodium phosphate dibasic heptahydrate, and LP solution were supplied by Sigma (St. Louis, MO). Hydrogen peroxide, concentrated nitric acid, and ferric nitrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$) were purchased from Macron Chemicals (Center Valley, PA).

2.2. Thiocyanate extraction

Fifteen grams of mustard meal were ground for 30 s using a Scienceware Micromill (Bel-art Products, Pequannock, NJ). Residual oil in a portion of the meal was extracted using the method of Borek and Morra (2005). Two grams of both the oil-containing mustard meal (MM) and defatted meal were added to separate 40-mL aliquots of deionized water. Mustard meal solutions were placed on an orbital shaker (VWR-S-500, San Francisco, CA) and the speed was set at 100 rpm for 3 d. Samples were centrifuged at 2000 g for 20 min or until supernatants obtained were clear. The supernatants were decanted into glass vials for the measurement of SCN^- .

To determine the effect of temperature on extraction efficiency, separate 2.0 g samples of mustard seed meal were added to 40 mL of deionized water and incubated in a shaking water bath at 30, 60, 70, and 90 °C for 30 min. Samples were removed at 10-min intervals, centrifuged, and SCN^- concentrations measured.

2.3. Measurement of lactoperoxidase activity

The method of Pruitt and Kamau (1994) as detailed by Campbell, Kang, Bastian, and Drake (2012) was used to measure LP activity. Potassium phosphate buffer (0.1 M) adjusted to pH 6.0 served as a solution to dissolve LP and to make the ABTS solution (1 mM). Three milligrams of lyophilized LP were dissolved in 10 mL of potassium phosphate buffer. To measure the activity, dilutions were made to maintain the absorbance within the linear range of the spectrophotometer. Two milliliters of the ABTS solution and 0.1 mL of the LP solution were added to a disposable cuvette with a 1-cm path length. The reaction was started by adding 1 mL of 3.2 mM H_2O_2 to the

solution. The reaction was monitored for 10 min, absorbance recorded at 412 nm, and results expressed as micromoles of ABTS oxidized per minute (U). A UV/Visible spectrophotometer with “UV Winlab” software (Perkin Elmer PTP6., San Jose, CA) was used to measure SCN^- , OSCN^- , and LP activity.

2.4. Lactoperoxidase system

A method by Adolphe and others (2006) for optimization of the LPSys was followed using SCN^- supplied from mustard meal extracts or KSCN. This comparison was to determine if additional compounds in mustard meal extracts affect the behavior of SCN^- in the LPSys. The protocol involved adding 250 μM H_2O_2 to 3.92 U/mL LP in a final volume of 50 mL of solution buffered at pH 6.0 (sodium phosphate buffer). The concentration of SCN^- varied from 125 to 1000 μM , and OSCN^- and SCN^- concentrations were measured at 0, 20, 40, 60, and 120 min. The concentration of SCN^- that generated the greatest amount of OSCN^- was used in assays with concentrations of H_2O_2 ranging from 500-1500 μM . To create sustained production of H_2O_2 , exogenous H_2O_2 was generated in-situ using glucose oxidase (GOx) and glucose. Concentrations of glucose were varied from 0 to 64 mM with a fixed GOx activity of 85.5 U/L. The glucose concentration that maximized OSCN^- production was determined and finally the level of GOx was increased 2 and 4 times to further increase OSCN^- production. The system capable of producing the highest concentration of OSCN^- was regarded as optimum. All of the experiments were repeated with MM- SCN^- and KSCN for comparison purposes.

2.5. Ion chromatography

Ion chromatography (IC) was used to verify accuracy of the more routinely used colorimetric method. To quantify SCN^- concentration using IC, the samples were filtered using 0.45- μm filters (Millipore, MA, USA) followed by 0.1- μm syringe filters (Millipore, MA, USA), and diluted as

required. Concentrations of SCN^- were quantified based on an external calibration curve. Ion chromatographic analysis was performed using a Dionex Ion Analyzer equipped with a GP40 gradient pump, ED40 electrochemical detector, and an AS40 auto sampler. Chromatographic separation was achieved on a Dionex Ion-Pac AS11 (250 mm×4mm) column using 100 mM NaOH as the mobile phase at a flow rate 0.5 mL/min. The overall run time was 10 min with SCN^- elution occurring at 5.3 min. The injection volume was 20 μL . The detector stabilizer temperature was set at 30 °C with temperature compensation of 1.7%/°C. Anion suppressor current was set to 300 mA.

2.6. Spectrophotometric method

Routine measurement of SCN^- was performed according to the method of Pruitt, Adamson, and Arnold (1979) using spectrophotometric analysis of an Fe-SCN complex at 460 nm. A stock solution of ferric nitrite was prepared by dissolving 10 g of ferric nitrate ($(\text{FeNO}_3)_3 \cdot 9\text{H}_2\text{O}$) in 20 mL of concentrated nitric acid and diluting with water to a final volume of 200 mL. To measure SCN^- , 0.5 mL of the LPSys was added to a 1 mL ferric nitrate stock solution and the absorbance was recorded with ferric nitrate as a reference. The sample absorbance was determined by comparison with an external standard curve obtained using KSCN concentrations ranging from 0.0625 to 1.0 mM.

2.7. Hypothiocyanite (OSCN^-) production

The production OSCN^- was measured according to the methods of Thomas and others (1994) and Bosch et al. (2000) as modified by Min, Krochta, and Rumsey (2007). Twenty milligrams of Nbs_2 was dissolved in 50 mL of ice-cold phosphate buffer (pH 7.2) containing 0.1 mM DETAPAC. The mixture was reduced with 1 mL of a freshly prepared solution of 42 μL 2-mercaptoethanol in 20 mL water to form a 0.6 mM Nbs solution. An aliquot of the LPSys (0.5

mL) was added to the cuvette containing 0.5 mL of cold buffered sodium chloride (pH 7.2) followed by introduction of 125 µL of the 0.6 mM Nbs solution. The absorbance at 409 nm was recorded using deionized water as a blank. The OSCN⁻ concentration was calculated using equation 1:

$$[OSCN^-] = \frac{(A_s - A_c) \times R_{f/s}}{0.01405 \times 2} \quad (1)$$

Where:

A_s: absorbance of the sample at 409 nm (A₄₀₉)

A_c: absorbance for control at 409 nm

R_{f/s}: the ratio of final and initial volumes (2.25 in this case)

0.01405: extinction coefficient of Nbs at 409 nm (µM/cm) (Bosch et al., 2000)

For the mustard meal-derived LPSys, the absorbance of the aqueous solution of LP and the mustard seed meal extract (before adding or generating H₂O₂) in phosphate buffer (pH 6.0) was added to the value of A_c.

2.8. Statistical analysis

All data presented represent the means of analyses performed in triplicate. The treatment effect on each of the tested parameters was determined by analysis of variance (ANOVA) using SAS version 9.0 (SAS, Cary, NC) and a p ≤ 0.05. Differences among group means were determined with the Least Square Mean test.

3. Results and Discussion

3.1. Extraction of SCN⁻

Results from conventional extraction of SCN⁻ from MM and defatted MM by shaking each in water for 3 d showed that removing residual oil from mustard meal had no effect (p<0.05) on the concentration of SCN⁻ extracted (defatted MM: 152±1.6 µM/g and MM: 155±4.2 µM/g).

Therefore, mustard meal with an oil content of approximately 10% by weight was used as the SCN⁻ source in subsequent experiments.

Yields of SCN⁻ determined at increasing temperatures from 30 to 90 °C indicated that myrosinase was active up to 70 °C and that maximum SCN⁻ concentration reached approximately 145 µmol/g meal (Table 1). Changes in the activity of myrosinase from *S. alba* in the presence of 3.38 mM ascorbic acid and 0.15 mM magnesium chloride in Bis-Tris buffer solution (pH 6.5) were shown by Van Eylen, Indrawati, Hendrickx, and Van Loey (2006) to increase up to 60 °C, with the enzyme experiencing deactivation at higher temperatures. Stability of myrosinase up to 70 °C in systems containing *S. alba* meal possibly results from increased complexity afforded by the inclusion of mustard meal as compared to the buffered solution used by Van Eylen, et al. (2006). Therefore, incubation at 70 °C with shaking was chosen as the method for production of SCN⁻ for all subsequent assays.

3.2. Hypothiocyanite production with increasing concentrations of SCN⁻

Thiocyanate generated by shaking water-meal mixtures at 70 °C for 10 min was used as one of the components to create an LPSys in which H₂O₂ (250 µM final concentration) was added exogenously. At the range of SCN⁻ concentrations tested in this experiment, the highest OSCN⁻ concentration (74 µM) was achieved with 500 µM SCN⁻ (p<0.05) and no readily explainable trends with time and SCN⁻ concentration were observed (Figure 1). For comparison purposes, KSCN was used in place of seed meal extracts in the LPSys, yielding lower maximum OSCN⁻ concentrations of 64 µM for all KSCN concentrations ≥ 250 µM (Figure 1). Concentration decreases in OSCN⁻ from the maxima were similar among treatments regardless of the starting KSCN concentration.

Differences in OSCN⁻ production between the two SCN⁻ sources occur as result of the

inclusion of a variety of compounds in the mustard meal extract in addition to SCN^- . These constituents may have effects on both the production and stability of OSCN^- by 1) protecting LP against inactivation, 2) providing additional substrate for OSCN^- production, and 3) supplying antioxidants that aid in the preservation of OSCN^- . Our data indicate that mustard meal extracts containing 500 μM SCN^- allow for maximum OSCN^- production and that other constituents in the extract have no or a slight positive effect on such production.

3.3. Hypothiocyanite production with increasing concentrations of exogenous H_2O_2

In an attempt to generate additional OSCN^- , higher concentrations of H_2O_2 were tested at fixed concentrations of LP and SCN^- (500 μM). When H_2O_2 concentration was above 500 μM , OSCN^- concentration decreased at the initial 10-min measurement in both the MM and KSCN systems (Figure 2). Our data are in agreement with previously reported results showing that in an LPSys with 50-100 μg of LP and 2-5 mM of SCN^- , maximum concentration of OSCN^- was produced when equimolar concentrations of H_2O_2 and SCN^- were used (Pruitt & Tenovuo, 1982). Decreased concentrations of OSCN^- result from the enhanced decay rate of OSCN^- as caused when molar H_2O_2 concentrations exceed those of SCN^- (Tenovuo et al., 1986).

An LPSys can thus be generated with MM extracts containing SCN^- , but rapidly generated OSCN^- decays quickly when H_2O_2 is supplied exogenously. Our results are in agreement with Tenovuo, et al. (1986) who used an LPSys containing KSCN (1 mM) and H_2O_2 (1 mM) at 37 °C to study the rate of SCN^- and H_2O_2 consumption and OSCN^- production. They recorded a rapid reduction in SCN^- concentration and production of $\text{HOSCN}/\text{OSCN}^-$ within the first minute of reaction initiation. After the first minute, there was little change in the concentration of $\text{HOSCN}/\text{OSCN}^-$ throughout the time period of the experiment (2 min). This suggests that the LPSys reaches equilibrium within the first few minutes and that OSCN^- decomposition occurs thereafter.

The decomposition of HOSCN/OSCN⁻ may result from its interaction with SCN⁻ (Thomas, 1981) or through self-decomposition in which one molecule of OSCN⁻ destroys three additional molecules of OSCN⁻, ultimately producing CO₂, NH₄⁺, and SO₄²⁻ (Hoogendoorn et al., 1977).

3.4. Hypothiocyanite production using glucose and glucose oxidase

In an attempt to promote longer-term production of OSCN⁻ beyond the initial flush provided using H₂O₂, glucose and GOx were substituted into the LPSys. Glucose concentrations from 8 to 64 mM were tested to determine if additional OSCN⁻ could be produced in the LPSys with MM extracts. Sustained OSCN⁻ production was achieved in the 32 and 64-mM treatments for approximately 9 h (Figure 3). Unlike the MM-LPSys, the KSCN system showed sustained OSCN⁻ production for 8 h with only 8 mM glucose (Figure 3). The KSCN-LPSys was a pure system with production and degradation of OSCN⁻ being governed only by LPSys components. In contrast, higher glucose concentrations are required with MM extracts, because the extract contains a variety of compounds such as sinapine (Dubie, Stancik, Morra, & Nindo, 2013) that potentially interfere with reaction kinetics.

Even with increased glucose, OSCN⁻ concentrations of approximately 15 µM (Figure 3) were still far below the concentrations (74 µM) obtained using exogenous H₂O₂ and MM extracts (Figure 1). In order to increase H₂O₂ production, the highest concentration of glucose (64 mM) was chosen and concentrations of GOx were increased 2 and 4 times. Doubling the concentration of GOx doubled the concentration of OSCN⁻ in the MM-LPSys, generating a maximum of approximately 20 µM OSCN⁻ (Figure 4). With 4 times the GOx concentration, OSCN⁻ was increased proportionally to a maximum of approximately 40 µM. Similar to previous comparisons between MM extracts and KSCN (Figure 3), higher OSCN⁻ concentration of 60 µM were obtained with KSCN extracts because of fewer interfering constituents. However, higher

maximum OSCN⁻ concentrations (as measured with greater GOx concentrations) resulted in more rapid rates of OSCN⁻ disappearance with time in both MM and KSCN treatments (Figure 4), most likely through self-decomposition in which one molecule of OSCN⁻ decomposes three molecules of OSCN⁻ resulting in CO₂, NH₄⁺, and SO₄²⁻ (Hoogendoorn et al., 1977).

Thus at a fixed level of LP (4.92 U/mL), the optimized concentrations of components in the mustard meal-derived LPSys were 32-64 mM glucose, 342 U/L GOx, and 500 μM SCN⁻. Even though the LPSys has been studied extensively, there are few reports on system optimization. In one investigation, component concentrations of the LPSys optimized using response surface methodology for a fixed concentration of SCN⁻ (500 μM) at 25°C were 85.5 U/L GOx, 8 mM glucose, and 3.93 U/mL LP (Adolphe, et al., 2006). However, the optimization was not based on the reaction product (OSCN⁻), but on residual SCN⁻ concentration. In other studies, OSCN⁻ was not measured or the activity of GOx was not reported (Fweja et al., 2008; Hayashi et al., 2012; Seifu et al., 2004).

4. Conclusion

Thiocyanate (SCN⁻) was extracted from *S. alba* seed meal and used in a lactoperoxidase system to produce hypothiocyanite (OSCN⁻), thus demonstrating the potential to develop such a system using GRAS components instead of non-food grade KSCN. An optimized LPSys for a fixed LP concentration of 4.92 U/ml was 32-64 mM glucose, 342 U/L GOx, and 500 μM SCN⁻ based on the antimicrobial OSCN⁻ agent that was generated using SCN⁻ derived from *S. alba* seed meal. Other studies have shown that 5 μM OSCN⁻ is sufficient to kill *E.coli* and reduce the bacterial population by 10 fold within 2 h of incubation (Marshall & Reiter, 1980). Likewise, in other previous studies *Salmonella enteritidis* in carrot juice was reduced by 3.7 log units after 4 h

incubation with 125 μM OSCN⁻ (Touch, et al., 2004) and a bacteriostatic effect of OSCN⁻ on *Listeria monocytogenes* has also been reported (Adolphe, et al., 2006; El-Shenawy, Garcia, & Marth, 1990; Seifu et al., 2004). The optimized LPSys in this study may have bacteriostatic effects on *L. monocytogenes* in liquid foods or *E. coli* in several food matrices, including fresh produce. The LPSys can potentially be used in misting systems of produce sections of grocery stores to reduce the risk of *Listeria* and *E.coli*. It can also be generated in dry form and sprayed inside meat or chicken packages or on the surface of produce. Further evaluation on the use of the *S. alba*-derived SCN⁻ as a substrate in the LPSys is needed.

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Author Contributions

A. Golmohamadi performed the experiments, assisted with experimental details, and drafted the manuscript. I. Popova assisted with extraction and analysis of meal extracts. M.J. Morra provided major advising and manuscript revision, C.I. Nindo guided the design of the experiments, directed the research, and edited the manuscript.

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Figure 1.

Figure 2.

442 Figure 3.

447 Figure 4.

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Figure Captions:

Figure 1. Hypothiocyanate (OSCN^-) concentrations in lactoperoxidase systems supplied with mustard meal (MM) SCN^- (top) or KSCN (bottom) and incubated for 2-h at room temperature with 250 μM of exogenous H_2O_2 . Error bars exceed the symbol size only slightly and have been removed for clarity. Means are for three replicates and values that share the same letter at each time point are not significantly different ($p < 0.05$).

Figure 2. Hypothiocyanate (OSCN^-) concentrations in lactoperoxidase systems supplied with 500 μM of mustard meal (MM) SCN^- (top) or KSCN (bottom) and incubated for 2-h at room temperature with 500 - 1500 μM of exogenous H_2O_2 . Error bars that exceed the symbol size only slightly have been removed for clarity. Means are for three replicates and values that share the same letter at each time point are not significantly different ($p < 0.05$).

Figure 3. Hypothiocyanate (OSCN^-) concentrations in lactoperoxidase systems during an 8-h incubation at room temperature with 500 μM SCN^- , 85.5 U/L GO, and variable glucose concentrations (4-64 mM). Error bars represent standard errors of the mean for three replicates. Means that share the same letter at a single time point are not significantly different ($p < 0.05$).

Figure 4. Hypothiocyanate (OSCN^-) concentrations in lactoperoxidase systems containing 500 μM SCN^- from mustard meal (MM) or KSCN, 64 mM glucose, 3.92 U/mL LP, and variable concentrations of GO at room temperature. Error bars exceed the symbol size only slightly and have been removed for clarity. Means are for three replicates and values that share the same letter at each time point are not significantly different ($p < 0.05$).

472 Table 1. Change in SCN⁻ production (μmol/g meal) with time and temperature for 2 g mustard
 473 meal/40 mL water¹.

Temp. (°C)	Time (min)		
	10	20	30
30	17.0±1.4 ^a	31.0±1.9 ^b	40.4±2.4 ^c
60	106.8±5.9 ^a	130.6±2.1 ^b	145.8±4.6 ^c
70	132.8±1.9 ^a	140.3±2.1 ^b	144.8±2.1 ^c
90	16.8±2.6 ^a	32.7±3.1 ^b	47.2±2.6 ^c

474 ¹ Values are means of triplicates ± standard error. Means in the same row with the same
 475 letter are not significantly different (p<0.05).

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