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Systematic Development of Peptide-Based Biostimulants from Whey Protein Hydrolysates

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Abstract: Due to global warming, agricultural production systems are exposed to increasing abiotic stresses, which threaten an economically and environmentally sustainable food production. Innovative environmentally friendly solutions are needed to cope with climate-related risks and to reduce the use of high amounts of synthetic agrochemicals. A promising solution to foster sustainable agriculture involves the use of biostimulants. This strategy, however, is not adopted by the industry to a great degree due to the scarce number of reproducible effects observed in published studies as well as the lack of fundamental knowledge about their mode of action. Biostimulants are substances that positively impact plant physiology by enhancing growth, improving fruit quality, and increasing stress resilience. Among these, protein hydrolysates stand out as a particularly promising category. However, their precise mechanisms of action and the optimal conditions for their application are still not fully understood. This project aims to develop peptide-based biostimulants in a reproducible manner, ensuring their availability for use in plant model systems under strictly controlled conditions. To achieve this, various protein hydrolysates will be produced through the enzymatic hydrolysis of whey, a by-product of cheese production. These hydrolysates will be employed in subsequent studies within plant model systems.

Keywords: Biostimulation · Enzymatic hydrolysis · Hydrolysis degree · Whey

1. Introduction

The UN Environment Emissions Gap Report, released in 2020, states that despite a brief dip in carbon dioxide emissions caused by the COVID-19 pandemic, the world is still heading for a temperature rise of more than 3 °C by the end of this century.[1] This exposes global agricultural production systems to increasing abiotic stresses, which threatens economically and environmentally sustainable food production.

Novel, innovative environmentally friendly solutions are urgently needed in order to better cope with climate-related risks and reduce the need for the application of high amounts of synthetic agrochemicals. A promising solution for a more sustainable way of agriculture involves the use of biostimulants. However, the use of biostimulants in agriculture is still at its infancy due to the lack of systematic reproducible studies and the lack of fundamental knowledge about their mode of action.

Biostimulants are natural substances, which can have positive effects on general plant physiology, thereby increasing biomass production, nutrient uptake, crop quality and yield as well as abiotic and biotic stress tolerance.[2,3] A promising class of biostimulants which has been shown to improve plant performance, fruit quality and stress resilience are protein hydrolysates.[4] However, the exact mechanisms of action and the optimal treatment conditions, such as concentrations, application frequency and composition of protein hydrolysates remain to be elucidated. Further studies are required to confirm and foster observed effects and to elucidate the mode of action of protein hydrolysates on plant physiology. This would help to promote their usage by the industry thereby fostering sustainable, environmentally friendly agronomic strategies in the context of global warming and the necessity to reduce agrochemicals.

The present project aims to produce various protein hydrolysates from whey concentrate in order to study their effects on plant model systems under precisely controlled conditions in subsequent studies. Whey can be used directly as a fertilizer with minimal or no harm to the environment and can be directly applied to soil, resulting in positive effects on soil properties and plant development.[5] Whey serves as an excellent nitrogen source for plant production, with its constituents playing a crucial role in fertilization and promoting microbiological growth in the soil.[6] Additionally, the use of whey enhances the physical and chemical properties of the soil.[7] In studies on biostimulant activity whey protein hydrolysates are of interest and the quantity of hydrolysates applied will be far below the concentration required for fertilization or soil quality improvement. For monitoring the progression of protein hydrolysis, the well-established TNBS method will be used.[8]

2. Materials and Methods

2.1 *Chemicals and Reagents*

Lactose, L-tryptophan, glycine, L-leucine, SDS, and stearic acid were obtained from Fluka Analytical (Darmstadt, Germany). Boric acid, phosphoric acid, sulfuric acid, sodium carbonate, sodium bicarbonate, and sodium hydroxide were acquired from Acros Organics (Geel, Belgium). Sulfuric acid for Kjeldahl titration (Titripur) was obtained from Supelco Analytical (Darmstadt, Germany). Kjeldahl tablets (without Hg/Se) were purchased from Merck (Darmstadt, Germany). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was sourced from Sigma-Aldrich (Darmstadt, Germany). Deionized water was obtained from a Milli-Q purification system (Millipore AG, Zug, Switzerland).

2.2 *Whey Preparation*

The raw milk was purchased from the Fromagerie d'Etiez, AOP & Artisan Cheese in Vollèges, Switzerland (fromagerie-etiez.ch). Raclette cheese production was carried out in the pilot plant of HES-SO Valais-Wallis, Institute of Life Technologies. The whey obtained after cheese production was centrifuged (CLARA 20 separation unit, Alfa Laval: 50 °C, 11130 G, 1.3 bar, flowrate 100 L/h) and then pasteurized (plate heat exchanger Rosista APV: 72 °C, 30 s). To increase protein content and decrease mineral and lactose levels, the centrifuged and pasteurized whey was ultrafiltered twice using a pilot unit (Type SW25 MMS, Tami membrane UF 3 kDa: 50 °C, TMP 1.5–2.5 bar, feed pump 400 L/h, flux 8–9 L/m²h). In the first ultrafiltration, 20 L of whey was concentrated to 1.5 L. For the second ultrafiltration, the 1.5 L from the first ultrafiltration were diluted with 20 L of demineralized water

and reconcentrated to 2.7 L under the same conditions as before. This whey concentrate was stored at –18 °C prior to use in hydrolysis studies.

2.3 *Whey Characterization*

2.3.1 *HPLC Analysis of Lactose*

An Agilent 1220 Infinity series liquid chromatograph (Agilent Technologies, CA, USA) comprised of an auto-sampler, a binary pump and a G1362A RI detector (Agilent Technologies 110 Series, Agilent Technologies, CA, USA) was employed for the chromatographic analysis as previously described.[9] Briefly, 5 µL were injected onto an amino column (Aminex HPX-87H Ion exclusion, 300×7.8 mm i.d., particle size 5 µm, Bio-Rad, Hercules, CA, USA) equipped with a precolumn (30×4.6 mm i.d., cation H cartridge for amino column). The mobile phase was composed of 5 mmol/L sulfuric acid and was delivered in an isocratic mode at a constant flow rate of 0.5 mL/min. The column temperature was set to 35 °C. Quantification of lactose was done with a refractive index detector. Lactose was quantified by external calibration.

2.3.2 *Protein Content*

The protein content of the whey samples was determined according to Kjeldahl method ISO 8968-3:2007/IDF 20-3: 2007 using a nitrogen-to-protein conversion factor of 6.38.[10] In contrast to the ISO method, quantification of ammonia was done by potentiometric titration with 0.25 mol/L sulfuric acid. Tryptophan and glycine served as reference compounds.

2.4 *Proteases*

The enzymes were generously provided by Novozymes AG (Bagsvaerd, Denmark) for the enzymatic hydrolysis of concentrated whey protein. For this study, we employed the endopeptidase Alcalase® Pure 2.4 L and the exopeptidase Flavourzyme® 1,000 L, both individually and in combination. Product data sheets are available from Novozymes.[11,12]

2.5 *Whey Protein Hydrolysis*

Several hydrolyses experiments have been done. In the present article, only three of them are shown.

1. *Hydrolysis: Alcalase®*

The whey concentrate (900 mL, corresponding to 8.3 g protein) was incubated with 450 mg Alcalase® (43.2 Anson units/g = 4.86 Anson units = 2673 IU)[13] = 360 µL Alcalase® enzyme preparation. A carbonate/bicarbonate buffer (90 mL 0,1 mol/L, pH 8.0) was added, and temperature was kept at 55 °C. During hydrolysis, pH goes down. With an automated pH controller (FerMac 260 Module, Electrolab, Gloucestershire, UK) pH Controller, the pH was kept constant at 8.0 with NaOH (1 mol/L).

2. *Hydrolysis: Flavourzyme® and Alcalase®*

The whey concentrate (900 mL, equivalent to 8.3 g of protein) was incubated with 225 mg of Flavourzyme (225 LAPU) = 180 µL of Flavourzyme enzyme preparation and 225 mg of Alcalase® (21.6 Anson units/g = 4.86 Anson units = 2673 IU)[13] = 180 µL of Alcalase® enzyme preparation. A carbonate/bicarbonate buffer (90 mL, 0.1 mol/L, pH 7.5) was added, and the temperature was maintained at 50 °C. The pH was kept at 7.5 using NaOH (1 mol/L) with an automated pump.

3. *Hydrolysis: Flavourzyme® and Alcalase®*

The whey concentrate (400 mL, equivalent to 3.7 g of protein) was incubated with 100 mg of Flavourzyme (100 LAPU) = 80 µL of Flavourzyme enzyme preparation and 100 mg of Alcalase® (21.6 Anson units/g = 2.16 Anson units = 1,188 IU)[13] = 80 µL of Alcalase® enzyme preparation. A carbonate/bicarbonate buffer (40 mL, 0.1 mol/L, pH 8.0) was added, and the temperature was maintained at 55 °C. The pH was kept at 8.0 using NaOH (1 mol/L) with an automated pump.

Sampling for all hydrolyses was done at 0 h, 0.5 h, 1 h, 1,5 h, 2 h, 4 h, 6 h, 8 h, 24 h to follow degree of hydrolysis. For each time point, 1 mL was taken and heated to 100 °C for 10 min in a thermo-block to terminate the enzyme reaction. After cooling, the degree of hydrolysis was determined with the TNBS method.

To evaluate the biostimulant activity of the whey hydrolysate on plant models, 200 mL samples were collected at 2, 4, 8, and 24 h. For hydrolysis 3, a 400 mL sample was collected only after 24 h. These samples were heat-inactivated before being stored at –18 °C for subsequent biostimulant testing.

2.6 *TNBS Method to Determine the Hydrolysis Degree*

The degree of hydrolysis was quantified using the TNBS method according to Adler-Nissen,[5] as described by Hernández-Herrero[14] with modifications. Aliquots (10 µL) of the unhydrolyzed whey protein solution (amino nitrogen content: AN_{UHWP}) were mixed with 80 µL of sodium phosphate buffer (0.2 mol/L, pH 8.5) and 80 µL of 0.02% (w/v) TNBS in a 96-well microplate. The hydrolysed samples (AN_{HWP}) were diluted (100, 200 and 500x) with the sodium phosphate buffer (0.2 mol/L, pH 8.5), then 90 µL of the dilutions were mixed with 80 µL of 0.02% (w/v) TNBS in a 96-well microplate. Microplates were incubated at 45 °C, and after 1 h, absorbance was measured at 420 nm using a plate reader (Biotek Synergy HT, Winooski, VT, USA). Leucine, at concentrations ranging from 0 to 100 mg/L (0–0.762 mmol/L), was used as a standard to determine the free amino group content of the samples (AN, in mg N/mg sample). The degree of hydrolysis (DH) was calculated using the following formula:

$$\%DH = (AN_{HWP} - AN_{UWP})/Npb \times 100$$

A value of 123.3 mg/g was used as the nitrogen content of the peptide bonds (Npb) for whey protein.[15] Each sample was analyzed in triplicate.

3. Results and Discussion

The whey obtained from raclette cheese production from raw milk was centrifuged. The obtained whey was twice ultrafiltered to concentrate the protein content and to decrease the presence of small molecules like minerals and lactose. After the second ultrafiltration step, the whey concentrate contained 9.2 g/L of protein and lactose content was reduced from 41.2 g/L to under 0.6 g/L.

The whey concentrate was hydrolyzed using proteolytic enzymes provided by Novozymes AG to generate protein hydrolysates for testing in plant models. The selection of an enzyme for a specific application depends on the substrate and the desired characteristics of the resulting hydrolysate. The objective of this study was to produce various hydrolysates for subsequent evaluation of their biostimulant activity in follow-up investigations. This paper presents the results obtained using the enzymes Alcalase® and Flavourzyme®. The optimal hydrolysis conditions for the broad-spectrum endopeptidase Alcalase® are a pH of 8 and a temperature range of 50–60 °C.[16] The exopeptidase Flavourzyme®, which also exhibits endopeptidase activity, has an optimal pH range of 6–8 and operates effectively at 90 °C.[16]

The degree of hydrolysis, characterized using the well-established TNBS method, served as an indicator of the protein hydrolyses. This analysis proved highly effective for monitoring the progression of protein hydrolysis. Other methods of monitoring the degree of hydrolysis during protein hydrolysis have been described in the literature, like pH-stat, osmometry, soluble nitrogen content, and the *o*-phthaldialdehyde (OPA) method.[17] In this ar-

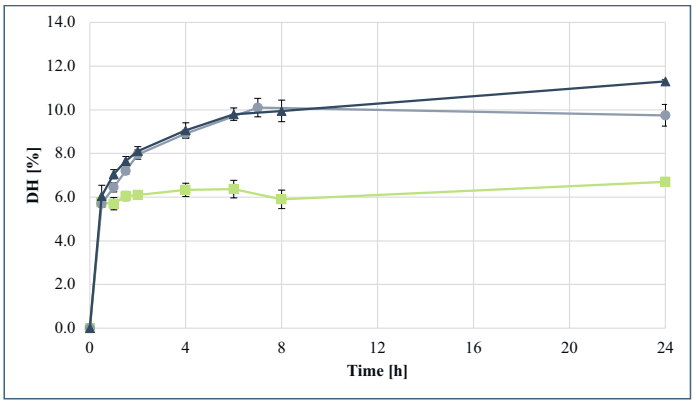

Fig. 1. Degree of hydrolysis (DH) after enzymatic hydrolyses of whey protein concentrate:
Hydrolysis 1: Alcalase® at pH 8,0 and 55 °C (■);
Hydrolysis 2: Alcalase® + Flavourzyme® at pH 7,5 and 50 °C (•);
Hydrolysis 3: Alcalase® + Flavourzyme® at pH 8,0 and 55 °C (▲).

ticle, we present the hydrolysis degrees of three distinct hydrolysates. Fig. 1 illustrates the time course of these hydrolyses. After 1 h, hydrolysis with the endopeptidase Alcalase® was nearly complete, reaching a degree of hydrolysis of approximately 6%. As anticipated, the combination of Alcalase® with the exopeptidase Flavourzyme® resulted in a higher degree of hydrolysis, around 10%. Small variations in pH and temperature had minimal impact on the degree of hydrolysis in the experiments with the combination of Alcalase® and Flavourzyme®. The degree of hydrolysis is strongly influenced by the choice of enzymes, pH, temperature, and the duration of hydrolysis. A molecular weight profile obtained through size exclusion chromatography, or an LC-MS analysis could provide a more comprehensive understanding of the structural differences among the resulting hydrolysates. However, this analysis falls outside the scope of the current study, which focused on producing diverse hydrolysates for biostimulation testing.

4. Conclusions

The enzymes Alcalase® and Flavourzyme® are appropriate proteolytic enzymes to produce protein hydrolysates from whey concentrate. The TNBS method facilitated the monitoring of the progression of protein hydrolysis. First screening experiments for the biostimulant activity of the whey hydrolysates are under investigation. The protein hydrolysate after 4 h of hydrolysis with Alcalase® and Flavourzyme® at 50 °C and pH 7.5 in an algae model showed promising results, to be confirmed in further studies.

Conflict of interest

The authors have no conflict of interest to report.

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