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Uncovering the Protein Conversion Potential of Alfalfa (*Medicago sativa* L.) and Duckweed (*Lemna minor* L.) Through Enzymatic Hydrolysis and Digestibility Assessment

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Abstract

The growing demand for sustainable protein alternatives has increased interest in underutilized plant biomasses with high nutritional potential. This study investigated the conversion efficiency of alfalfa (*Medicago sativa* L.) and duckweed (*Lemna minor* L.) proteins through multienzyme hydrolysis, with the aim of evaluating how carbohydrate–protein matrix interactions influence enzymatic accessibility and apparent protein digestibility. Three biotechnological hydrolysis schemes were applied, involving combinations of α-amylase, amyloglucosidase, protease, pepsin, pancreatin, and bile salts, including an in vitro gastrointestinal digestion simulation. The first hydrolysis scheme demonstrated that starch-rich matrices formed a viscous medium that reduced protease mobility and limited protein cleavage. Improved substrate accessibility was achieved when plant material was pre-treated with amylolytic and proteolytic enzymes, which resulted in a noticeably higher release of free amino acids. Amino acid profiling revealed that this enzymatic sequence was the most effective for disrupting carbohydrate-associated protein fractions in both species. In vitro digestion assays indicated higher apparent protein conversion for duckweed compared to alfalfa under standardized laboratory conditions. Overall, the results confirm that appropriate multienzyme strategies can enhance amino acid liberation from complex plant matrices and highlight duckweed biomass as a promising candidate for sustainable protein valorization.

Keywords: amino acid profile; biomass valorization; enzymatic hydrolysis; in vitro apparent protein digestibility; plant protein

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1. Introduction

The growing global demand for sustainable protein resources has accelerated efforts to identify and develop alternative plant-based biomasses capable of supporting future food and feed systems. While plant protein technologies are increasingly recognized as an essential part of modern biotechnology, their role in meeting the rapidly rising global need for protein is still developing [1]. Even though plant-based proteins are widely available and generally cheaper than animal-derived alternatives, they are not yet used directly in human diets to the extent one might expect. Most plant-based proteins are instead funneled into animal feed, where they serve as inputs for producing meat, milk, eggs, and other animal-based foods. However, this route is highly inefficient: livestock are able to convert only a small fraction, roughly 3%, of the plant protein they consume into animal protein [2].

As a result, the traditional model of producing protein through animals shows substantial biochemical and resource-related inefficiencies.

Recent reviews emphasize that aquatic plants such as duckweed (*Lemna minor* L.) present substantial potential as alternative protein sources due to their rapid growth, high protein content, and minimal cultivation inputs (e.g., land and fertilizer), making them attractive candidates for bioeconomy solutions [3]. Under optimized cultivation conditions, duckweed can reach protein contents of 30–40% dry matter and has been shown to provide a balanced amino acid profile with amino acid scores comparable to or exceeding those of conventional plant proteins [4–8]. In addition, its capacity for nutrient recovery from agro-industrial effluents further enhances its sustainability appeal. Nevertheless, despite these advantages, the integration of duckweed into existing value chains remains limited, partly due to technological, economic, and regulatory uncertainties. Moreover, structural properties of duckweed cell walls and the presence of phenolic–protein complexes can impair protein accessibility and enzymatic hydrolysis efficiency, leading to variability in reported bio-accessibility estimates across studies [9,10].

In parallel, leaf-derived proteins from terrestrial crops such as alfalfa (*Medicago sativa* L.) represent another promising but structurally distinct protein source. Alfalfa remains one of the most productive temperate forage crops, delivering high biomass yields along with crude protein contents typically ranging from 18% to 25% of dry matter [11,12]. Recent developments in green biorefinery systems have demonstrated that alfalfa can be efficiently fractionated into leaf protein concentrates and protein-rich juices that show potential for use in monogastric feed and food applications [13]. For instance, protein extraction from its leaf-based biomass offers a high yield of leaf protein concentrate; however, structural matrix complexity (fiber and polysaccharides) can impede extraction efficiency [14]. Moreover, despite its high protein density, a substantial proportion of alfalfa protein remains structurally embedded within the fiber–chloroplast matrix, limiting both extraction efficiency and protein accessibility during digestion [15]. As a result, the effective nutritional value of alfalfa protein is often lower than that predicted by crude protein content alone. Therefore, advancing the use of alternative leafy or aquatic biomasses requires not only exploring their compositional advantages but also addressing key challenges related to extraction efficiency, fractionation technologies, and product functionality. Understanding these structural constraints is crucial for enabling the transition from traditional crop-based proteins toward more diverse and resilient protein sources aligned with future sustainability goals.

Taken together, these examples highlight that the effective utilization of alternative leafy and aquatic biomasses requires not only consideration of their compositional advantages but also a clearer understanding of the structural constraints that influence protein accessibility. Addressing these matrix-related limitations is, therefore, essential to support the transition from conventional crop-based proteins toward more diverse, efficient, and resilient protein sources aligned with future sustainability objectives.

As interest in sustainable, plant-based protein sources continues to expand, research activity has become increasingly interdisciplinary, integrating molecular biology, analytical chemistry, environmental sciences and applied biotechnology. Consequently, visual scientometric tools such as keyword co-occurrence networks offer a valuable means of revealing how these domains interact, where research priorities are converging, and which conceptual pathways most strongly influence advances in protein extraction and functional characterization. The keyword co-occurrence network generated using VOSviewer, based on the term plant protein extraction, reveals a complex and interconnected research landscape in which plant-derived proteins are situated at the intersection of multiple scientific domains. Although plant proteins constitute the conceptual core of the network, the surrounding clusters demonstrate that their study extends far beyond simple composi-

tional analysis and is increasingly influenced by advances in biochemistry, cellular biology, environmental science, and biotechnology. The blue cluster, which centers around gene expression, metabolism, and plant developmental processes, reflects the growing emphasis on understanding how protein accumulation and composition are regulated at the molecular level. Such integration is essential for identifying plant species and tissues with favorable protein profiles or biochemical traits that facilitate extraction, hydrolysis, or downstream biotransformation. The green cluster encompasses chemical and physicochemical analyses, including protein purification, nutritional evaluation, and the characterization of plant biomolecules. These analytical components are particularly relevant when working with structurally complex plant biomasses—such as alfalfa or duckweed—in which proteins are embedded within fibrous or carbohydrate-rich matrices that may limit extraction efficiency. The red cluster, dominated by biomedical and pharmacological terms, highlights the role of plant proteins and peptides in therapeutic, functional, or physiological studies. Although this cluster may appear distant from traditional protein extraction research, its proximity to terms like isolation and purification, protein expression, and bioactivity suggests that extracted plant proteins are increasingly valued not only as nutritional components but also as bioactive molecules with potential applications in human health. The yellow cluster highlights environmental and microbiological contexts, including biomass utilization, microbial transformation, and bioremediation. Low-input, rapidly growing plant species such as duckweed are frequently examined within this cluster due to their potential as renewable protein sources, especially in circular bioeconomy frameworks (Figure 1).

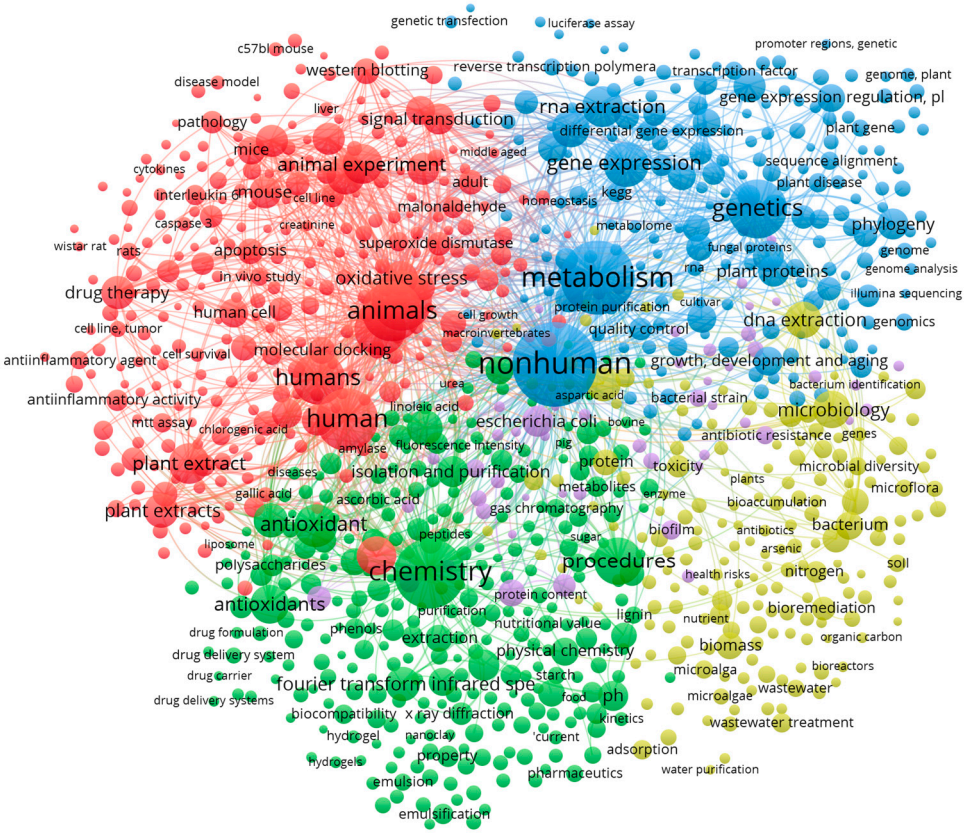

Figure 1. Co-occurrence network of keywords.

Within this evolving research landscape, investigating how enzymatic strategies improve protein recovery and amino acid release from underutilized biomasses such as alfalfa and duckweed directly aligns with the emerging scientific priorities depicted in the network. The increasing interest in sustainable protein sourcing, biochemical conversion techniques,

and plant-based nutritional optimization further underscores the relevance of the present study. Enzymatic hydrolysis has therefore become a key strategy for improving protein liberation and digestibility in structurally complex green biomasses. Enzyme-assisted fractionation has been shown to enhance protein solubility, disrupt polysaccharide–protein interactions, and increase the release of low-molecular-weight peptides [16,17]. Despite these advances, no comprehensive comparative analyses have evaluated alfalfa and duckweed under unified enzymatic hydrolysis and digestibility frameworks. Existing studies typically investigate one biomass type in isolation, employ heterogeneous processing conditions, or assess digestibility using non-standardized in vitro protocols. This fragmentation prevents a meaningful understanding of their relative protein conversion potential and limits their integration into scalable protein-producing biorefinery systems. To bridge this gap in understanding, our study examines how efficiently proteins from alfalfa and duckweed can be converted through carefully designed multienzyme hydrolysis approaches, placing particular emphasis on how the structural features of their plant matrices affect the release of amino acids and the overall digestibility of the resulting protein fractions.

2. Materials and Methods

2.1. Materials

Alfalfa (contained 11.8% crude protein, 3.1% crude fat, 58.6% total fiber, and 1.17% starch) cultivar "Neptune", grown in Lithuania in 2020, was obtained from the agricultural cooperative ŽŪB "Lašai". The cultivar was developed by *Semences de France* and registered in 2008 (France). Duckweed (contained 22.0% crude protein, 8.1% crude fat, 45.4% total fiber, and 8.2% starch), belonging to the Lemnaceae family, was cultivated and harvested in the Netherlands.

The plant materials were air-dried and ground to a particle size of $\leq$1 mm using a laboratory mill at the Food Research Centre of the Food Institute, Kaunas University of Technology (Kaunas, Lithuania).

For enzymatic hydrolysis, commercial enzyme preparations were obtained from *Megazyme* (UK). Thermostable α-amylase from *Bacillus licheniformis* was used for starch hydrolysis, protease from *Bacillus licheniformis* for protein hydrolysis, and amyloglucosidase from *Aspergillus niger* for the hydrolysis of α-1,4 and α-1,6 glycosidic bonds in oligosaccharides. The enzyme dosage was 50–100 g per 100 kg of substrate, and the optimal pH and temperature conditions are summarized in Table 1.

Table 1. Enzymes and their optimal conditions for hydrolysis.

Enzyme	Enzyme Activity	Optimal pH	Optimal Temperature, °C
Thermostable α-amylase, 3000 U/mL	Amylolytic	7	100
Purified protease, 350 U/mL	Proteolytic	7	60
Purified amyloglucosidase, 3300 U/mL	Amyloglucosidase	4	60

Moreover, for pH adjustment during enzymatic hydrolysis, MES/TRIS buffer solution, 0.56 N hydrochloric acid (HCl), and 5% sodium hydroxide (NaOH) solution were used, all purchased from Sigma-Aldrich (Merck, Darmstadt, Germany) and used as analytical grade.

For the in vitro simulation of gastrointestinal digestion, enzyme preparations were obtained from Sigma-Aldrich (Steinheim, Germany). Human salivary α-amylase was used for carbohydrate hydrolysis, porcine pepsin for protein hydrolysis under acidic conditions, and porcine pancreatin, containing trypsin, amylase, and lipase, for the digestion of proteins, carbohydrates, and lipids. Bile salts were added to maintain physiological ionic strength and optimal enzyme activity. The enzymes and their optimal activity conditions are summarized in Table 2.

Table 2. Enzymes used for In Vitro gastrointestinal digestion and their optimal conditions.

Enzyme	Enzyme Activity	Optimal pH	Optimal Temperature, °C
α-Amylase, 300–1500 U/mg	Amylolytic	7	37
Pepsin, 3200–4500 U/mg	Proteolytic	3	37
Pancreatin, 8*USP	Amylolytic, Lipolytic, Ribonucleolytic, Proteolytic	7	37
Bile salts	For pancreatin activation	7	37

For the simulation of gastrointestinal conditions, various buffer and salt solutions were prepared using analytical-grade reagents obtained from Sigma-Aldrich (Steinheim, Germany), including potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), ammonium carbonate ($(NH_4)_2CO_3$), calcium chloride ($CaCl_2$), sodium hydroxide (NaOH), and hydrochloric acid (HCl). All solutions were prepared with distilled water and used for pH adjustment and to mimic the ionic environment of the gastrointestinal tract.

2.2. Removal of Lipids from Plant Materials

Alfalfa flour (45 g) was mixed with diethyl ether (250 mL) in a 500 mL conical flask, shaken at 250 rpm for 1 h at room temperature, then filtered and spread on sterile Petri dishes. Samples were left in a fume hood for 24 h to evaporate residual solvent. Duckweed flour (10 g) was treated similarly with 100 mL diethyl ether. The dried samples were weighed, and the amount of removed lipids was determined by the weight difference. Defatting was performed to minimize the influence of lipids on enzymatic hydrolysis and protein hydrolysis assessment, allowing a more focused evaluation of protein–carbohydrate matrix interactions during digestion.

2.3. Determination of Chemical Composition of Plant Materials

The chemical composition of plant materials was determined using standard methods relevant for interpreting enzymatic hydrolysis results. Lipid content was measured by acid hydrolysis (AOAC 922.06 and AOAC 963.15:2003/1K:2013). Nitrogen and crude protein content were determined by the Kjeldahl method (ISO 20483:2014), with protein calculated by multiplying nitrogen by 6.25. Total dietary fiber was measured using the Megazyme total dietary fiber kit by the enzymatic–gravimetric method (AOAC 985.29, 1990). Starch content was determined according to the validated procedure of the Lithuanian Ministry of Agriculture (Order No. 3D-145, Vilnius, 2003-04-08).

2.4. Enzymatic Hydrolysis of Plant Materials

Enzymatic hydrolysis of plant materials was performed using three approaches. The first evaluated the effect of thermostable α-amylase and amyloglucosidase on protease activity. The second compared the hydrolysis efficiency of the enzymatic system across different plant materials. The third assessed the hydrolysis of two plant materials using an in vitro system simulating human gastrointestinal digestion.

2.4.1. Effect of Thermostable α-Amylase and Amyloglucosidase on Protease Activity

This method was designed to assess whether starch hydrolysis by amylases influences subsequent enzymatic protein hydrolysis by proteases. Two 400 mL conical flasks were prepared, each containing 2 g of defatted alfalfa flour. Alfalfa was selected as a representative raw material to evaluate whether prior starch hydrolysis by thermostable α-amylase and amyloglucosidase affects subsequent protease activity. As the objective of this experiment was to examine potential enzymatic interactions rather than to compare different biomasses, only a single raw material was used for this preliminary assessment. To each flask, 80 mL of MES-TRIS buffer was added, and the flour was dispersed using a magnetic stirrer. Stirring speed was then reduced, and 100 µL of thermostable α-amylase was added to one flask. Both flasks were covered with foil and incubated in a water bath at 98–100 °C for 30 min, then cooled to 60 °C. The pH was adjusted to 4.5 using 0.56 N HCl. The flask containing α-amylase was then supplemented with 400 µL of amyloglucosidase, and the reaction continued at 60 °C for 30 min. After hydrolysis, the pH of both flasks was adjusted to 7.5 using 5% NaOH, and 200 µL of protease solution was added to each flask. Flasks were incubated at 60 °C for 30 min to allow protein hydrolysis. Samples were then transferred for analysis of free amino acids. The experiment was repeated to ensure reproducibility. Hydrolysis efficiency, expressed as the percentage of protein converted to free amino acids, was calculated using Equation (1).

$$x = \frac{m_{fa}}{m_b \times m_1} \times 100 \quad (1)$$

where m_{fa}—mass of free amino acids in the sample solution (g/mL of solution), m_b—protein content per gram of sample (g); m_1—mass of the sample (g).

2.4.2. Enzymatic Hydrolysis Efficiency of the Different Plant Materials

This method aimed to eliminate the influence of acidic and alkaline hydrolysis and to compare the hydrolysis efficiency of equal amounts of protein from different plant materials. Two 400 mL conical flasks were prepared with 0.5 g of duckweed flour each, and two flasks with 1 g of defatted alfalfa flour each. To each flask, 40 mL of MES/TRIS buffer was added, and the mixture was dispersed using a magnetic stirrer. Stirring speed was then reduced, and 50 µL of thermostable α-amylase was added to one flask of each plant material. Flasks were covered with foil and incubated in a water bath at 98–100 °C for 30 min, then cooled to 60 °C. While stirring, 100 µL of protease solution was added to all flasks, and hydrolysis was performed at 60 °C for 30 min. Flasks were then opened, and the pH was adjusted to 4.5 using 0.56 N HCl. Flasks containing α-amylase were supplemented with 200 µL of amyloglucosidase and incubated for 30 min at 60 °C. After hydrolysis, samples were transferred for analysis of free amino acids. The experiment was performed in triplicate to confirm reproducibility.

After determining the amount of free amino acids, the hydrolysis efficiency (the percentage of protein converted to free amino acids) was calculated using Equation (1).

2.4.3. In Vitro Gastrointestinal Digestion Simulation

The in vitro gastrointestinal digestion procedure implemented in this study was adapted from established static digestion models previously employed to simulate human gastrointestinal conditions [18,19]. This protocol involves sequential oral, gastric, and intestinal phases with controlled pH adjustments, physiologically relevant enzyme solutions, and bile salts to approximate key features of human digestion. While not a direct implementation of the INFOGEST consensus model, the approach follows recognized in vitro digestion methodologies and ensures comparability with existing studies on plant protein bio-accessibility.

Preparation of Simulated Fluids

Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared by combining the volumes of stock solutions listed in Table 3 in a 500 mL flask and diluting with water to the mark. The solutions were mixed, and the pH was adjusted using 6 M HCl to 7.0 for SSF, 3.0 for SGF, and 7.0 for SIF.

Table 3. Composition of simulated fluids.

Component (mL)	SSF (pH 7.0)	SGF (pH 3.0)	SIF (pH 7.0)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	6.8	12.5	42.5
NaCl	0.0	11.8	9.6
$MgCl_2{\cdot}6H_2O$	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.06	0.5	0.0
HCl	0.09	1.3	0.7

Preparation of Enzyme Solutions

***Pepsin solution (25,000 U/mL)*:** Pepsin (0.06250 g) was weighed, transferred to a 10 mL volumetric flask, and diluted to volume with simulated gastric fluid (SGF).

Pancreatin solution: Pancreatin (0.5 g) was weighed, transferred to a 50 mL volumetric flask, and diluted to volume with simulated intestinal fluid (SIF).

***Bile salt solution (160 mM)*:** Bile salts (1.675 g) were weighed, transferred to a 25 mL volumetric flask, and diluted to volume with simulated intestinal fluid (SIF).

Sample Preparation

Four samples were prepared. Two 100 mL conical flasks were each loaded with 2 g of defatted duckweed flour, while the remaining two flasks were each loaded with 4 g of defatted alfalfa flour. Different sample masses were used to standardize protein content across the samples.

The in vitro method was designed to simulate human gastrointestinal digestion in three sequential phases (oral, gastric, and intestinal) by using appropriate enzymes and by controlling salt composition, pH, and the duration of each digestive stage.

Simulation of the Oral Digestion Phase

Each sample was placed in a separate flask and mixed with 10 mL of simulated salivary fluid (SSF), homogenized with a glass rod, and supplemented with 0.5 mL of amylase solution, 25 µL of $CaCl_2$ solution, and 975 µL of distilled water. The flasks were incubated at 37 °C and 350 rpm for 2 min. Samples were then centrifuged (1000 rpm, 2 min), the

supernatant was discarded, and the pellet was washed three times with fresh SSF to remove residual enzymes.

Simulation of the Gastric Digestion Phase

Each sample was mixed with 7.5 mL of simulated gastric fluid (SGF), 1.6 mL of pepsin solution, 5 μL of $CaCl_2$ solution, 0.2 mL of 1 M HCl, and 695 μL of distilled water. The pH was adjusted to 3.0. The flasks were incubated at 37 °C and 350 rpm for 2 h. Samples were then centrifuged (1000 rpm, 2 min), the supernatant was discarded, and the pellet was washed three times with fresh SGF to remove residual enzymes.

Simulation of the Intestinal Digestion Phase

Samples were mixed with 11 mL of simulated intestinal fluid (SIF), 5 mL of pancreatin solution (trypsin activity: 800 U/mL), 2.5 mL of bile salt solution, 40 μL of $CaCl_2$ solution, 0.15 mL of 1 M NaOH, and 1.31 mL of distilled water. The pH was adjusted to 7.0 using 1 M NaOH. The flasks were incubated at 37 °C and 350 rpm for 2 h. Samples were then centrifuged (1000 rpm, 2 min), the supernatant was discarded, and the pellet was washed three times with fresh SIF to remove residual enzymes.

Sample Preparation for Protein Hydrolysis

After in vitro digestion, the samples were spread onto sterile Petri dishes and dried to constant weight at 60 °C. Portions of 0.2 g of dried duckweed and 0.4 g of dried alfalfa were placed into clean test tubes, and 25 mL of 6 M HCl was added. The tubes were sealed and incubated at 110 °C for 24 h to achieve complete hydrolysis of residual protein fractions. After hydrolysis, amino acids released from residual protein materials were quantified by high-performance liquid chromatography with fluorescence detection (UHPLC–FLD). Apparent protein digestibility was calculated as the proportion of protein-derived amino acids recovered after simulated gastrointestinal digestion followed by acid hydrolysis. Apparent protein digestibility, representing maximal protein conversion potential, was calculated using Equation (2).

$$x = \frac{m_{bh} - m_{ah}}{m_{bh}} \times 100 \quad (2)$$

where m_{bh}—protein content in the sample before digestion (g), m_{ah}—protein content remaining after simulated digestion and subsequent acid hydrolysis (g).

This approach quantifies apparent protein digestibility, reflecting the maximal protein conversion potential under laboratory conditions, rather than physiological in vivo digestibility, as residual protein fractions resistant to digestive enzymes are rendered quantifiable during the acid hydrolysis step.

2.5. Determination of Amino Acid Content by UHPLC—FLD

Non-oxidized samples of alfalfa and duckweed (0.2 g each) were hydrolyzed with 25 mL of 6 M HCl in 50 mL test tubes at 110 °C for 24 h. To prevent excessive pressure buildup during the initial stage, the tubes were loosely capped, then tightly sealed for the remainder of the hydrolysis.

After cooling to room temperature, the hydrolysates were carefully neutralized to pH 2.2 by gradual addition of 17 mL of 1 M sodium hydroxide solution while continuously stirring in the presence of 150–200 mL of citrate buffer, ensuring that the temperature did not exceed 40 °C. The neutralized mixtures were quantitatively transferred into 250 mL volumetric flasks and brought to the final volume with additional citrate buffer. Prior to chromatographic analysis, the solutions were filtered through 0.22 μm membrane filters to

remove particulates and ensure clarity of the sample, preventing potential blockages in the UHPLC system.

Amino acid separation was performed on a YMC-Triart C18 UHPLC column (1.9 µm particle size, YMC Co., Ltd., Kyoto, Japan) using a UFLC system (Shimadzu, Kyoto, Japan) equipped with an RF-20Axs fluorescence detector and an SIL-30AC automatic injector with pre-treatment functionality. The mobile phase consisted of solvent A (20 mmol/L potassium phosphate buffer, pH 6.5) and solvent B (acetonitrile/methanol/water, 45/40/15, $v/v/v$). The flow rate was set at 0.5 mL/min, and the column temperature was maintained at 45 °C. Fluorescence detection was carried out at excitation/emission wavelengths of 350/450 nm, switching to 266/305 nm after 9.0 min. Quantification was performed using external calibration curves prepared from amino acid standards (A9781, Sigma-Aldrich, Steinheim, Germany).

The amino acid content (g/100 g of product) was calculated using the following Equation (3).

$$x = \frac{M_a \times \frac{(V \times C_a)}{1{,}000{,}000} \times 100}{m_1} \quad (3)$$

where M_a—amino acid molar mass (g/mol), V—volume in which the sample was prepared (L) C_a—amino acid concentration (µmol/L), m_1—mass of the sample (g).

Experiments were performed in triplicates, and the obtained values were expressed as mean values ± standard deviations.

3. Results

3.1. Sample Preparation and Processing for Free Amino Acid Analysis

The experimental workflow used for sample preparation and pH adjustment prior to free amino acid determination is presented in Figure 2.

The schematic overview illustrates the sequence of extraction, buffering, pH modification, and sample handling steps applied to plant material before laboratory analysis. Plant samples were extracted using a MES–TRIS buffer solution under controlled conditions to ensure reproducibility. Following extraction, the samples were filtered to remove insoluble residues and divided into separate fractions for pH treatment. One set of extracts was adjusted to pH 4.5 using 0.56 N HCl, while another set was adjusted to pH 7.5 using 5% NaOH. After pH correction, the samples were incubated and mixed to allow stabilization of the adjusted conditions. Subsequently, the treated extracts were further processed and prepared for analytical measurements. All samples were then sent for laboratory analysis, where free amino acid content was determined. This experimental design enabled the evaluation of pH-dependent differences in free amino acid profiles, which are presented and discussed in the following sections.

3.2. Protein Hydrolysis Efficiency with/Without Amylases in Duckweed and Alfalfa

In the experimentally determined amino acid profile of duckweed (Table 4), the total amino acid content was 22.00 g/100 g. The highest levels were observed for aspartic acid (4.09 g/100 g) and alanine (3.29 g/100 g). Relatively high amounts were also detected for glutamic acid (1.65 g/100 g), leucine (1.17 g/100 g), phenylalanine (1.15 g/100 g), and proline (1.15 g/100 g). The remaining amino acids were detected at levels lower than 1 g/100 g.

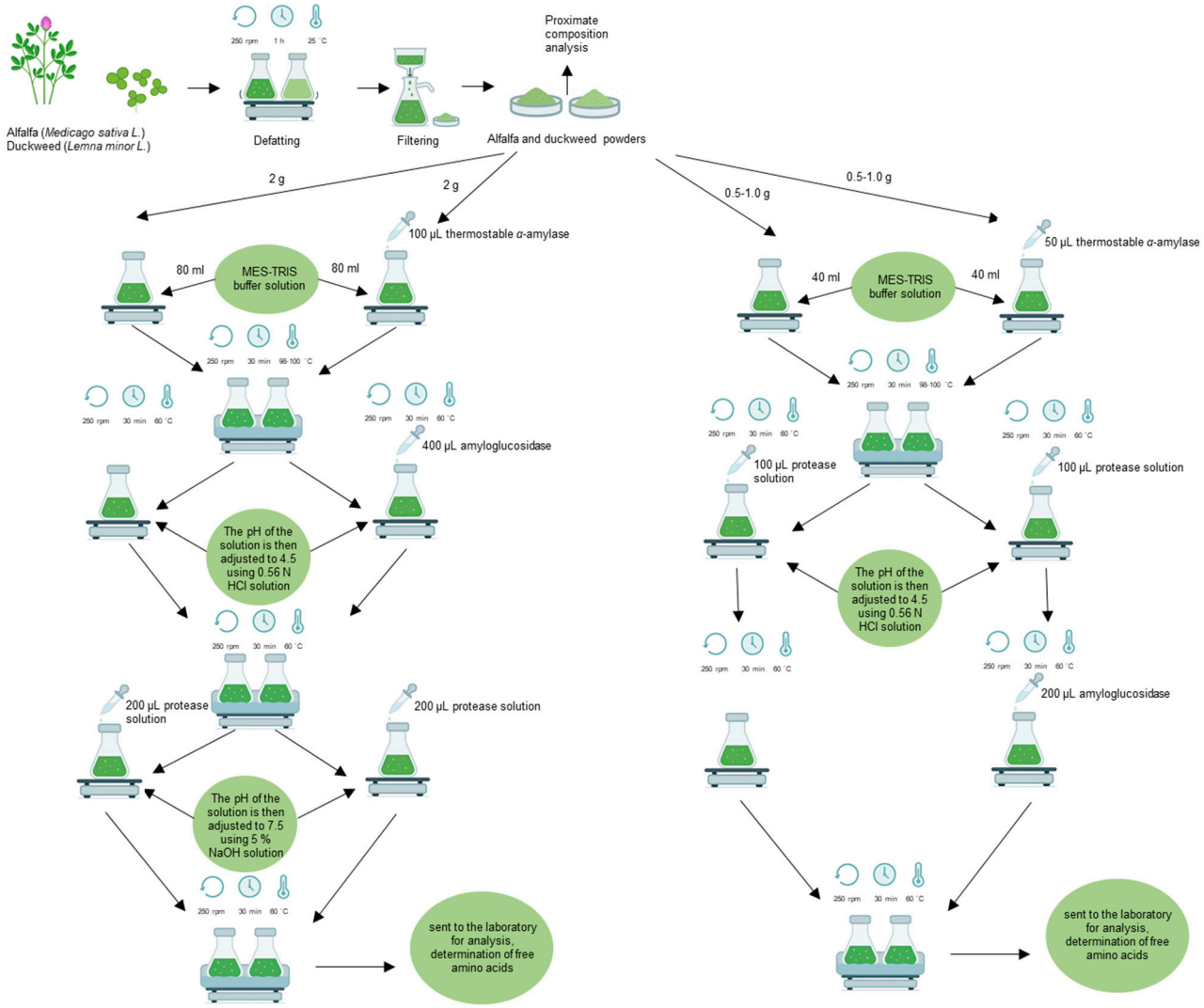

Figure 2. Schematic overview of sample preparation, pH adjustment, and processing steps applied prior to free amino acid analysis.

Table 4. Amino acid composition of duckweed protein and free amino acid profiles of duckweed samples with and without amylase treatment.

	Amino Acid Composition of Duckweed Proteins, g/100 g	Free Amino Acid Content of Duckweed/Without Amylases, g/100 g	Free Amino Acid Content of Duckweed/with Amylases, g/100 g
Aspartic acid (ASP)	4.09 ± 0.20 a	0.05 ± 0.00 b	0.16 ± 0.01 c
Glutamic acid (GLU)	1.65 ± 0.08 a	0.04 ± 0.00 c	0.41 ± 0.02 b
Serine (SER)	nd	0.02 ± 0.00	0.02 ± 0.00
Histidine (HIS)	0.72 ± 0.04 a	0.04 ± 0.00 b	0.06 ± 0.00 b
Glycine (GLY)	0.20 ± 0.01 a	0.01 ± 0.00 b	0.01 ± 0.00 b
Threonine (THR)	0.74 ± 0.04 a	0.02 ± 0.00 b	0.03 ± 0.00 b
Arginine (ARG)	0.81 ± 0.04 a	0.03 ± 0.00 b	0.04 ± 0.00 b
Alanine (ALA)	3.29 ± 0.16 a	1.63 ± 0.08 b	1.81 ± 0.09 b
Tyrosine (TYR)	0.89 ± 0.04 a	0.01 ± 0.00 b	0.01 ± 0.00 b

Table 4. *Cont.*

	Amino Acid Composition of Duckweed Proteins, g/100 g	Free Amino Acid Content of Duckweed/Without Amylases, g/100 g	Free Amino Acid Content of Duckweed/with Amylases, g/100 g
Cysteine (CYS)	0.51 ± 0.03 a	0.18 ± 0.01 b	0.15 ± 0.00 b
Valine (VAL)	0.59 ± 0.03 a	0.09 ± 0.01 b	0.08 ± 0.00 b
Methionine (MET)	0.71 ± 0.04	nd	nd
Tryptophan (TRP)	0.50 ± 0.03 a	0.06 ± 0.00 b	0.06 ± 0.00 b
Phenylalanine (PHE)	1.15 ± 0.06 a	0.003 ± 0.00 b	0.003 ± 0.00 b
Isoleucine (ILE)	0.74 ± 0.04 a	0.06 ± 0.003 b	0.06 ± 0.00 b
Leucine (LEU)	1.17 ± 0.06 a	0.10 ± 0.00 b	0.10 ± 0.00 b
Lysine (LYS)	0.76 ± 0.04 a	0.17 ± 0.01 b	0.19 ± 0.01 b
Proline (PRO)	1.15 ± 0.06	nd	nd
Total:	22.00 ± 1.10 a	2.52 ± 0.13 c	3.19 ± 0.16 b

Note: nd—not detected. Different lowercase letters (a–c) indicate statistically significant differences in amino acid concentrations between duckweed samples with and without amylase treatment ($p < 0.05$).

The total free amino acid content was 2.520 g/100 g in samples without amylase treatment and increased to 3.192 g/100 g after amylase application. Alanine was the predominant free amino acid in both treatments (1.634 and 1.814 g/100 g, respectively). Among the essential amino acids, leucine, lysine, and valine were the most abundant both before and after amylase treatment. Following amylase application, the concentrations of glutamic acid increased from 0.042 to 0.405 g/100 g and aspartic acid from 0.046 to 0.160 g/100 g; lysine also increased slightly from 0.170 to 0.191 g/100 g, whereas leucine remained unchanged at 0.104 g/100 g. In contrast, cysteine decreased from 0.184 to 0.154 g/100 g, and valine from 0.091 to 0.079 g/100 g. The histidine content increased from 0.039 to 0.058 g/100 g (Δ = 0.019 g/100 g). For the remaining amino acids, the differences between treatments did not exceed 0.005 g/100 g. Methionine and proline were not detected under either treatment.

In the experimentally determined amino acid profile of alfalfa (Table 5), the total amino acid content was 11.82 g/100 g. The highest concentrations were observed for aspartic acid (1.30 g/100 g) and glutamic acid (1.20 g/100 g), followed by leucine (0.83 g/100 g), proline (0.79 g/100 g), phenylalanine (0.67 g/100 g), tyrosine (0.65 g/100 g) and isoleucine (0.65 g/100 g). The remaining amino acids were detected at levels lower than 0.6 g/100 g.

Table 5. Amino acid composition of alfalfa protein and free amino acid profiles of alfalfa samples with and without amylase treatment.

	Amino Acid Composition of Alfalfa Proteins, g/100 g	Free Amino Acid Content of Alfalfa/Without Amylases, g/100 g	Free Amino Acid Content of Alfalfa/ with Amylases, g/100 g
Aspartic acid (ASP)	1.30 ± 0.07 a	0.08 ± 0.00 b	0.09 ± 0.01 b
Glutamic acid (GLU)	1.20 ± 0.06 a	0.03 ± 0.00 b	0.04 ± 0.00 b
Serine (SER)	nd	0.03 ± 0.00	0.04 ± 0.002
Histidine (HIS)	0.56 ± 0.03 a	0.01 ± 0.00 b	0.02 ± 0.00 b
Glycine (GLY)	0.15 ± 0.01 a	0.01 ± 0.00 b	0.01 ± 0.00 b

Table 5. *Cont.*

	Amino Acid Composition of Alfalfa Proteins, g/100 g	Free Amino Acid Content of Alfalfa/Without Amylases, g/100 g	Free Amino Acid Content of Alfalfa/ with Amylases, g/100 g
Threonine (THR)	0.55 ± 0.03 a	0.03 ± 0.00 b	0.03 ± 0.00 b
Arginine (ARG)	0.52 ± 0.03 a	0.02 ± 0.00 b	0.03 ± 0.00 b
Alanine (ALA)	0.56 ± 0.03 a	0.05 ± 0.00 b	0.06 ± 0.00 b
Tyrosine (TYR)	0.65 ± 0.03 a	0.01 ± 0.00 b	0.01 ± 0.00 b
Cysteine (CYS)	0.36 ± 0.02 a	0.02 ± 0.00 b	0.03 ± 0.00 b
Valine (VAL)	0.30 ± 0.02 a	0.04 ± 0.00 b	0.05 ± 0.00 b
Methionine (MET)	0.48 ± 0.02	nd	nd
Tryptophan (TRP)	0.31 ± 0.02 a	0.04 ± 0.00 b	0.04 ± 0.00 b
Phenylalanine (PHE)	0.67 ± 0.03 a	0.03 ± 0.00 b	0.03 ± 0.00 b
Isoleucine (ILE)	0.65 ± 0.03 a	0.03 ± 0.00 b	0.04 ± 0.00 b
Leucine (LEU)	0.83 ± 0.04 a	0.01 ± 0.00 b	0.02 ± 0.00 b
Lysine (LYS)	0.55 ± 0.03 a	0.03 ± 0.00 b	0.04 ± 0.00 b
Proline (PRO)	0.79 ± 0.04 a	nd	0.01 ± 0.00 b
Total:	11.82 ± 0.59 a	0.47 ± 0.02 c	0.60 ± 0.03 b

Note: nd—not detected. Different lowercase letters (a–c) indicate statistically significant differences in amino acid concentrations between alfalfa samples with and without amylase treatment ($p < 0.05$).

The analysis of free amino acids showed that the total free amino acid content was 0.472 g/100 g in samples without amylase treatment and increased to 0.599 g/100 g after amylase application. This increase was accompanied by higher post-treatment concentrations of several individual amino acids: aspartic acid content increased from 0.081 to 0.091 g/100 g, alanine from 0.052 to 0.063 g/100 g, valine from 0.043 to 0.051 g/100 g, leucine from 0.014 to 0.021 g/100 g, histidine from 0.006 to 0.018 g/100 g, glutamic acid from 0.028 to 0.035 g/100 g, serine from 0.034 to 0.040 g/100 g, arginine from 0.016 to 0.027 g/100 g, cysteine from 0.023 to 0.032 g/100 g, tryptophan from 0.035 to 0.043 g/100 g, isoleucine from 0.034 to 0.042 g/100 g, and lysine from 0.034 to 0.043 g/100 g. Within the essential amino acid fraction, valine, tryptophan, isoleucine, and lysine were the most abundant both before and after treatment. In contrast to these increases, methionine remained at 0.000 g/100 g in both treatments, whereas proline was not detected in untreated samples but reached 0.006 g/100 g following amylase treatment. For the remaining amino acids not listed above, the differences between treatments did not exceed 0.005 g/100 g.

4. Discussion

4.1. Total Amino Acid Profile Comparison of Duckweed and Alfalfa

The amino acid analysis conducted in this study demonstrates that duckweed (*Lemna minor*) possesses a high total amino acid content (22.00 g/100 g), with aspartic acid and alanine representing the most abundant amino acids. Essential amino acids, notably leucine, lysine, and valine, were also detected at considerable levels, indicating a nutritionally valuable protein composition. This profile coincides with previously published data showing that duckweed species typically contain substantial proportions of both essential and non-essential amino acids, with reported distributions of approximately 39.20% essential, 53.64% non-essential, and 7.13% non-proteinogenic amino acids [20]. Moreover, branched-chain amino acids, particularly leucine, valine, and isoleucine, have repeatedly been reported

among the dominant amino acids in the protein fraction of *L. minor* grown under different cultivation conditions. Taken together, these compositional features are consistent with comparative studies showing that the essential amino acid profile of duckweed is not only comparable but, in some cases, exceeds conventional legumes or other plant protein sources, and closely aligns with World Health Organization recommendations for indispensable amino acids [21]. Overall, these observations reinforce the classification of duckweed as a high-quality plant protein source and highlight its strong potential for food, feed, and biotechnological applications [22].

In contrast, alfalfa exhibited lower total amino acid levels than duckweed in the present study; however, the obtained values fall within the ranges reported in the literature. Previous scientific investigations have documented that total or crude protein contents in alfalfa biomass typically range from approximately 16 to 30 g/100 g, depending on the plant fraction and processing method, with corresponding variation in amino acid concentrations [23]. In this work, the total amino acid content measured in alfalfa (11.82 g/100 g) falls at the lower end of these reported ranges but remains broadly consistent with the literature. It has also been shown that alfalfa leaves contain both essential and non-essential amino acids and display generally consistent amino acid profiles across various cultivars and plant tissues. In agreement with our findings, aspartic acid and glutamic acid are most often reported as the predominant amino acids in alfalfa, while leucine, lysine, valine, and phenylalanine are present at nutritionally required levels [24]. Although alfalfa proteins are considered nutritionally balanced, the higher total amino acid content and greater representation of essential amino acids observed in duckweed highlight its superior protein density and amino acid richness. Considering that structurally complex in plant biomasses, protein accessibility is governed by the overall carbohydrate–protein matrix architecture, including structural polysaccharides (cellulose, hemicellulose), lignocellulosic barriers, and potential phenolic compounds and protein interactions. A more detailed fractionation and compositional characterization of these matrix components would be valuable to further disentangle their individual contributions to enzymatic efficiency. Such investigations could provide deeper mechanistic insight into how biomass structure modulates protein conversion potential.

4.2. Impact of Enzymatic Treatment on Duckweed and Alfalfa Proteins

Plant proteins are generally recognized to have lower digestibility compared with animal-derived proteins, which more closely resemble human proteins in both structural characteristics and amino acid composition [25]. This limitation highlights the importance of optimizing processing strategies to fully exploit the nutritional potential of plant-based proteins. In this context, enzymatic treatment of plant cells has emerged as an effective approach to enhance protein accessibility and increase the release of free amino acids. In the present study, free amino acid levels in both duckweed and alfalfa were measured before and after enzymatic treatment, revealing that such processing can significantly improve protein hydrolysis and the availability of nutritionally important amino acids, thereby supporting the potential of enzymatic methods to enhance the functional and nutritional quality of plant proteins. In both duckweed and alfalfa, application of amylases resulted in increased concentrations of free amino acids, indicating effective protein breakdown into smaller peptides and free amino acids. The results show that after enzymatic treatment, the free amino acid content of duckweed and alfalfa increased by approximately 21%. This outcome is consistent with numerous scientific publications demonstrating that enzyme-assisted hydrolysis and extraction enhance the release of free amino acids and peptides from plant protein matrices through peptide bond cleavage and partial degradation of cell wall components, thereby improving protein solubilization and accessibility [26,27]. For

example, this effect have been documented for duckweed, where Bernier et al. reported that enzymatic hydrolysis of *Lemna minor* using proteases such as pepsin, chymotrypsin, papain, and trypsin achieved degrees of hydrolysis of up to approximately 9% without prior protein extraction, highlighting the high susceptibility of duckweed proteins to enzymatic degradation and the efficient release of peptides and amino acids [28]. Comparable trends have also been described for alfalfa; in this scenario, Youngmi Kim et al. demonstrated that the use of cell wall-degrading enzymes (e.g., pectinases and cellulases) in combination with alkaline extraction significantly improved protein recovery from alfalfa leaves and produced protein fractions with amino acid compositions comparable to soy protein isolates, supporting the effectiveness of enzymatic systems in enhancing amino acid release [18]. Overall, studies across diverse plant protein sources confirm that enzymatic hydrolysis under mild conditions increases the formation of free amino acids and small peptides, thereby improving protein accessibility, enzymatic hydrolysis efficiency, and functional properties. This is consistent with the enzyme-induced free amino acid increase observed in the present study.

4.3. In Vitro Apparent Digestibility of Duckweed and Alfalfa Proteins: Interpretation and Perspectives

In vitro gastrointestinal digestion models are widely applied to assess the potential bio-accessibility of plant-derived proteins, particularly in cases where structural complexity and matrix composition may limit enzymatic accessibility [19]. Such models allow for controlled evaluation of protein breakdown under simulated gastrointestinal conditions while minimizing biological variability inherent to in vivo studies [29]. It is difficult to find comparative studies on the protein digestibility of alfalfa and duckweed; therefore, comparative analyses often rely on separating and synthesizing results from individual raw material studies [30–33]. In the present study, a standardized three-phase in vitro gastrointestinal digestion protocol (oral, gastric, and intestinal) was applied to compare the apparent protein digestibility of duckweed and alfalfa, two structurally distinct plant biomasses, with particular emphasis on the behavior of proteins embedded in carbohydrate-rich plant matrices. To ensure comparable nitrogen input during in vitro digestion, sample masses were adjusted according to crude protein content, following an iso-nitrogenous design. It should be noted, however, that crude protein normalization does not account for potential differences in protein composition or structural conformation between biomasses, which may influence digestion kinetics.

High apparent protein digestibility values were observed for both materials under the applied experimental conditions. The corresponding values for duckweed and alfalfa following simulated gastrointestinal digestion are summarized in Table 6.

Table 6. Apparent protein digestibility of duckweed and alfalfa after in vitro gastrointestinal digestion.

Biomass	Apparent Protein Digestibility (%)
Duckweed	96.75 ± 0.21%
Alfalfa	94.11 ± 0.23%

At first glance, these values suggest extensive protein breakdown under simulated gastrointestinal conditions; however, their interpretation requires careful consideration of the analytical methodology applied. Apparent digestibility in this study was calculated based on total amino acid recovery following enzymatic digestion combined with subsequent acid hydrolysis of residual material. As a result, protein fractions resistant to gastrointestinal enzymes but remaining in the solid residue were chemically cleaved during acid hydrolysis and included in the final amino acid quantification. Consequently, the reported values repre-

sent maximal protein conversion potential rather than true physiological protein digestibility in vivo. Within this framework, the relatively small difference between duckweed and alfalfa reflects comparable protein accessibility under the applied laboratory conditions rather than definitive differences in nutritional digestibility. Accordingly, the digestion assay was used to characterize general protein conversion performance, providing a comparative basis for evaluating protein accessibility in structurally distinct plant biomasses and supporting further research on enzymatic processing and protein valorization.

It is important to consider that protein content and amino acid composition in both alfalfa and duckweed may vary depending on seasonal growth conditions, harvest stage, nutrient availability, and geographic location. Previous studies have demonstrated that environmental factors such as temperature, photoperiod, and cultivation system can influence biomass composition in leafy crops and aquatic plants [29]. While beyond the scope of the current study, a more detailed assessment of these environmental and cultivation-related factors would represent an important direction for future research and could substantially enhance the scientific value of this topic.

Although the present study focused on apparent protein digestibility under standardized in vitro conditions, the results highlight the broader importance of biomass structure and protein-matrix organization in determining protein accessibility. From this perspective, the present work provides a foundation for future studies addressing targeted fractionation, selective removal of non-protein components, and tailored enzymatic treatments aimed at improving protein recovery and functionality. Such approaches may enable more efficient integration of structurally diverse plant biomasses into sustainable protein production and valorization pathways.

5. Conclusions

This study provides a comparative evaluation of protein conversion potential in alfalfa and duckweed using multienzyme hydrolysis and standardized in vitro apparent protein digestibility assessment. The results demonstrate that both biomasses can be effectively processed through enzyme-assisted strategies, leading to enhanced protein accessibility and increased release of free amino acids from structurally complex plant matrices. In particular, the sequential application of amylolytic and proteolytic enzymes proved critical for disrupting carbohydrate–protein interactions and improving enzymatic efficiency. Duckweed exhibited a higher total amino acid content and slightly higher apparent protein conversion under in vitro digestion conditions compared to alfalfa. Nevertheless, alfalfa also demonstrated favorable protein conversion behavior when appropriate enzymatic pretreatment was applied. Together, these findings confirm that tailored multienzyme approaches can substantially improve protein utilization from underexploited plant biomasses.

Despite these promising outcomes, several limitations of the present study should be acknowledged. The enzymatic hydrolysis experiments were conducted under controlled laboratory conditions, and the in vitro digestion model, while standardized, cannot fully replicate the complexity of human gastrointestinal processes or account for inter-individual variability. Additionally, the study focused on overall amino acid release and digestibility, without addressing peptide size distribution, specific bioactive peptide formation, or functional properties such as solubility, emulsification, or sensory impact in final food systems. Future research should therefore expand toward scaling enzymatic processes, integrating targeted fractionation strategies, and evaluating techno-economic feasibility under industrially relevant conditions. Further investigations into peptide bioactivity, allergenicity, and nutritional performance in in vivo models would also strengthen the understanding of these biomasses as viable protein sources. Overall, this work contributes to the growing body of evidence supporting duckweed and alfalfa as promising components of sustainable

protein systems and provides a foundation for their further development within circular bioeconomy-oriented food and feed applications.

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