

**Title:** Enzyme-assisted Protein Isolation from Alfalfa Leaves

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**Abstract:**

Green leaves are recognized as one of the largest renewable sources of high quality protein for human and animal consumption. This work examines the use of commercially available, cell-wall degrading enzymes (Cellic CTec2, Viscozyme L, and Pectinex Ultra SP-L) for the extraction of proteins from alfalfa leaves. The enzyme-assisted, alkaline extraction of alfalfa protein using Viscozyme L and Pectinex Ultra SP-L enhanced the protein extraction by a factor of 1.4–1.6 at a low enzyme dose (< 6 mg/g dry alfalfa) as compared to the protein extraction without enzymes. The current work demonstrates the effect of pectinolytic and hemicellulolytic enzyme activities in these enzyme preparations in improving the protein extractability from alfalfa leaves and the quality of proteins obtained. The amino acid contents of extracted alfalfa leaf protein were comparable to those of isolated soybean protein reported in literature, except for glutamic acid and cysteine. The enzymatic hydrolysis of alfalfa leaf proteins using a protease (Alcalase) produced peptides with antioxidative activities as measured by the reducing power assay.

**1. Introduction**

Plant-based protein has received much attention in the past decade as an alternative source of protein due to the ever-growing demand for protein-rich foods and increased awareness of health and environmental issues associated with animal-protein [1]. Among the various sources of plant-based protein, green leaves are recognized as the most abundant and renewable sources of high-quality protein for human and animal consumption [2].

Alfalfa leaves are one of the most important raw leaf protein sources due to the high crude protein content and balanced amino acid composition ratio which is consistent with the Food and Agriculture Organization (FAO)'s recommended adult amino acid profile [3,4]. However, application of alfalfa proteins for human consumption has been limited by undesirable qualities, such as color, taste, and texture.

Alfalfa leaf proteins consist of an equal fraction of “white” and “green” proteins<sup>4</sup>. The white protein fraction has the benefit of high digestibility in humans, desirable functional properties such as excellent emulsification, heat stability and good water solubility. The green protein fraction lacks these positive qualities and has negative sensory properties [5,6]. Discarding green insoluble proteins would improve the sensory properties of alfalfa protein isolate for human consumption but at the cost of losing nearly half of the potential protein yield. Such limitations can be overcome by applying enzymatic hydrolysis to produce peptides that are easier to digest with improved solubility and sensory properties.

The protein from alfalfa also provides a source of high-value antioxidative properties [7]. Antioxidative peptides are low-molecular-weight polypeptides, comprising 2 to 20 amino acid residues, that exhibit various physiological functions [8]. The peptides produced from alfalfa leaf proteins exhibited not only high nutritive values but also good antioxidative properties comparable to reduced glutathione, a reference native antioxidant [7]. Food-derived antioxidative peptides have high stability and activity, ease of absorption, and cause no hazardous immunoreaction compared to antioxidant enzymes. Antioxidative peptides produced from alfalfa leaves can be used as functional foods, nutraceuticals, dietary supplements and constituents of pharmaceuticals.

The lack of extraction processes that achieve high protein yields has been recognized as one of the major obstacles in the production of alfalfa leaf proteins, especially on a commercial scale [9].

Achieving a high yield of proteins from alfalfa leaves is an important criterion to meet for economically feasible production of proteins. Most previous studies in alfalfa protein extraction from fresh leaves involved leaf juice extraction, protein precipitation by heat or acids, followed by protein separation. Final protein yield from alfalfa with respect to the initial protein are typically in the range of 15-60% depending on the extraction and protein coagulation techniques applied with varying ratios of green to white proteins [3,10]. Extracting food grade leaf proteins at high yields requires development of a suitable extraction process that targets both soluble and insoluble proteins and is mild enough to preserve amino acids.

The main goal of this study is to investigate the use of plant cell-wall degrading enzymes (cellulases, hemicellulases, and pectinases) in assisting the extraction of proteins from alfalfa leaves and to extend the use of alfalfa leaf proteins as a source of bioactive peptides. Treatment of alfalfa leaves with a proper combination of cell-wall degrading enzymes is expected to increase extractability of proteins by breaking down and exposing the intracellular structures and making plant cells more accessible during the subsequent protein extraction. In the case of wet distillers' grains, a similar approach achieved a protein-rich, fiber free enhanced DDGS indicating the efficacy of the cellulase enzymes in separating and preserving proteins in DDGS [11, 12]. However, investigation of the enzyme-assisted extraction of proteins from alfalfa leaves using these multi-component carbohydrases has not been previously reported.

In this study, three different commercial enzyme blends (Cellic<sup>®</sup> CTec2, Viscozyme<sup>®</sup> L, Pectinex<sup>®</sup> Ultra SP-L) were used, individually and in combination, to study the effect of these plant-cell wall degrading enzymes on the protein extractability from alfalfa leaves. Cellic CTec2, derived from *Trichoderma reesei*, is a cellulase complex specifically developed for the degradation of cellulose to fermentable sugars. It contains cellulases,  $\beta$ -glucosidases and hemicellulases that are necessary to hydrolyze cellulose fraction of plant cell walls into its constituent sugars. It has been widely utilized for the enzymatic saccharification and fermentation of various lignocellulosic materials into fuels and chemicals. Pectinex Ultra SP-L, a commercial enzyme derived from *Aspergillus aculeatus*, has a range of hemicellulolytic activities including polygalacturonase, cellulase,  $\beta$ -galactosidase, and fructosyltransferase, that can macerate and disrupt plant cells [13]. It is commonly used for the preparation of mash foods and extraction of juices and oils from fruits, vegetables and grain [14-16]. Viscozyme L is also a fungal enzyme complex containing a wide range of carbohydrolytic enzymes, such as arabanase, cellulase,  $\beta$ -glucanase, hemicellulase and xylanase, having activities against plant cell wall polysaccharides. This multi-component carbohydrase has wide applications in the processing of fruits, cereal, starch as well as in brewing industries. Viscozyme L has been used in protein extraction from various food materials, such as oat bran [17, 18], rice bran [19], and soybean flour [20]. These multi-component enzyme preparations are advantageous for cleaving the linkages within the plant cell wall matrix and hence improve the extraction of intercellular constituents such as sugars, oligosaccharides, as well as proteins.

The aims of this study are: 1) to study the effect of cell-wall degrading enzymes on protein extraction from alfalfa leaves and determine the optimal blend of enzymes that maximizes protein yields; 2) to measure yields and amino acid composition of the alfalfa protein obtained by the enzyme-assisted extraction and compare them to those produced by a non-enzymatic conventional protein extraction method; and 3) to measure the antioxidative property of peptides obtained from proteolytic enzymatic treatment of alfalfa proteins. The process diagram of enzyme-assisted protein extraction from alfalfa leaves is shown in Figure 1.

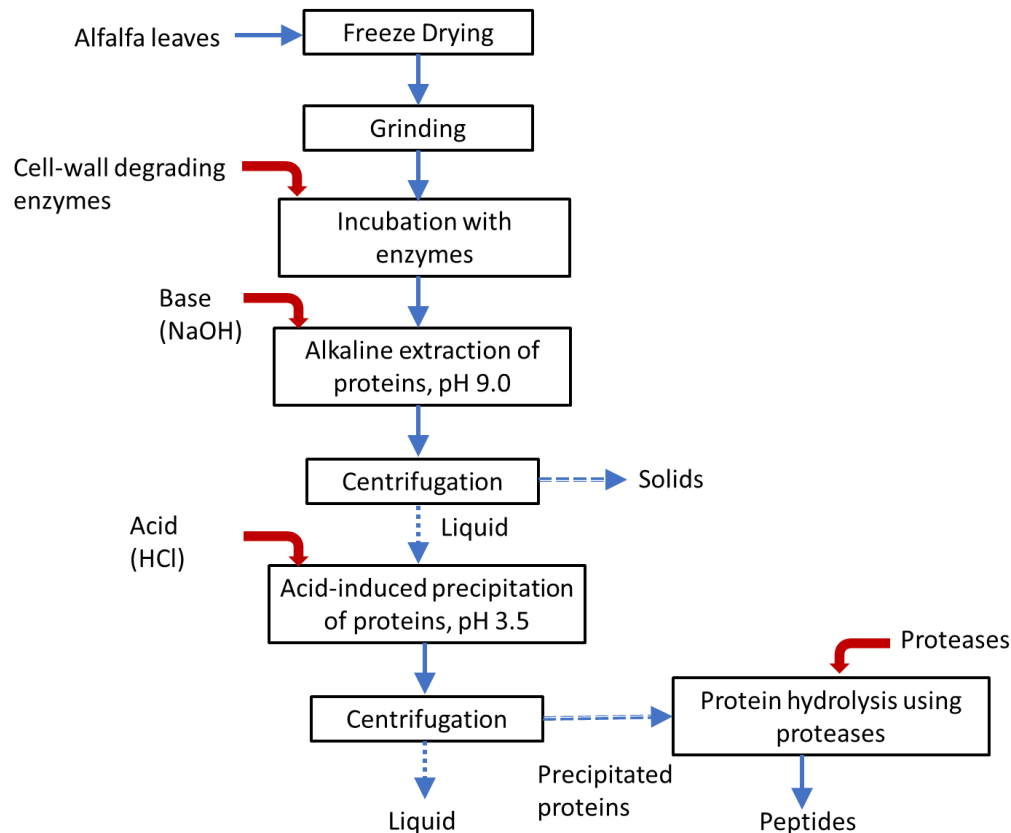


Figure 1. Schematic process diagram of enzyme-assisted alfalfa protein extraction and protein hydrolysis.

## 2. Materials

Fresh alfalfa leaves of the “Harv-extra” variety were harvested on the Nickin Silt Loam, 2 to 6 percent slopes soil type, located in Mann Valley Farm, River Falls, WI. The leaves were hand-picked and stored in a freezer. The frozen leaves were lyophilized at  $-50^{\circ}\text{C}$ , 0.2 mbar for 24 hrs and ground to fine powder ( $<1$  mm particles) using mortar and pestle. Dry leaves from alfalfa bales from the same location were also hand-picked and ground following the same procedure. The fresh and dry ground leaves were each mixed thoroughly and stored separately in zip-lock bags. The ground leaves were kept in a freezer until used. The alfalfa leaves used in this study contained  $30.7 \pm 0.1\%$  crude proteins,  $3.1 \pm 0.1\%$  crude fat,  $9.3 \pm 0.15\%$  crude fiber,  $9.7 \pm 0.1\%$  ash, and 47% other substances on dry basis. Enzymes, reagents, and chemicals, unless otherwise noted, were purchased from MilliporeSigma (St. Louis, MO).

## 3. Methods

All measurements described in Methods were made in duplicates or triplicates. Error bars represent standard deviation of the mean value.

### 3.1. Compositional Analysis

Crude protein and amino acid profile analyses of the samples were performed by the Experiment Station Chemical Laboratories, University of Missouri Columbia. Crude protein was determined as  $\%N \times 6.25$  by combustion analysis (LECO). Moisture content was determined after 24 hours of drying at  $105^{\circ}\text{C}$  in a convection oven.

### 3.2. Enzymatic treatment of alfalfa leaves

#### 3.2.1. Screening for an optimal enzyme blend

Cellic CTec2 (C), Viscozyme L (V), and Pectinex Ultra SP-L (P) enzymes (Novozymes Corp., Copenhagen, Denmark) were used in various combinations to degrade cell-wall structure of alfalfa leaves. Enzyme protein concentrations (mg protein/mL enzyme) were measured with the DC protein assay (Bio-Rad, CA) using BSA as a standard. Cellic CTec 2, Viscozyme L, and Pectinex Ultra SP-L contained  $173.5 \pm 24.5$  mg/mL,  $43.3 \pm 9.0$  mg/mL, and  $13.1 \pm 4.1$  mg/mL proteins, respectively. The enzymes, either individually or in various combinations, were added at different loadings (0 mg – 51 mg proteins/g dry leaves) to 25 mL Nalgene bottles containing 6.4 % (w/v, by dry weight) ground alfalfa leaves in pH 5.0 sodium citrate buffer. Each slurry was incubated for 24 hrs at 50°C, 150 rpm, followed by adding 0.5M NaOH to adjust pH to 9.0. The mixture was then incubated at 50°C, 150 rpm for 6 hrs. Then, each hydrolysate was centrifuged at 4500 rpm for 30 min. The supernatant was collected and analyzed for soluble proteins by using Bio-Rad DC protein assay kit. Corresponding control runs for each enzyme dose were prepared without alfalfa leaves. Extracted soluble protein yields were calculated by:

$$\% \text{ Protein Extracted} = \frac{SP_{\text{enzyme treated}} - SP_{\text{control}}}{\text{Total protein in alfalfa leaves (g)}} \times 100 \quad (1)$$

in which  $SP_{\text{enzyme treated}}$  = soluble protein in the supernatant of enzyme-treated alfalfa leaves (g);  $SP_{\text{control}}$  = soluble protein in the supernatant of control runs (without alfalfa leaves) (g).

The protein yields at different incubation period was also measured by following the same procedure described above. The enzyme mixture, Cellic CTec 2 (C), Viscozyme L (V), and Pectinex Ultra SP-L (P), combined at 1:1:1 volume ratio, was added to 6.4% (w/v by dry weight) alfalfa slurry at the enzyme dose of 51 mg protein/g dry alfalfa. At various time intervals (0, 0.5, 2, 8, 12, and 24 hrs) the enzyme treatment was terminated, followed by the protein solubilization at pH 9.0 using 0.5 M NaOH. After incubating for 6 hrs at 50°C, 150 rpm, supernatant was collected, centrifuged, and analyzed for the soluble protein concentration.

#### 3.2.2. Extraction and recovery of alfalfa proteins using the optimal enzyme blend

A 10% (w/v by dry weight) slurry of fresh, ground alfalfa leaves in pH 5.0 sodium citrate buffer was prepared in two 1 L flasks. To each slurry, 0.02% sodium azide was added to prevent microbial growth during the incubation. The enzyme mixture of Viscozyme L (V) and Pectinex Ultra SP-L (P), combined at 1:1 volume ratio, was added at the enzyme dose of 6 mg protein/g dry alfalfa. The slurries were incubated for 8 hrs at 50°C, 150 rpm, followed by adding 2M NaOH to adjust pH to 9.0. The mixture was then incubated at 50°C, 150 rpm for 6 hrs. The hydrolysates were then centrifuged at 4500 rpm for 20 min. The supernatant was decanted into another flask and adjusted to pH 3.5 using 1M HCl to precipitate proteins for 2 hrs. The precipitated solids were recovered and centrifuged at 4500 rpm for 5 min. The collected solids from centrifugation was lyophilized at -40°C for 24 hrs. A control run was identical as above except adding enzymes. The initial alfalfa leaves, remaining solids after protein extraction, and freeze-dried acid precipitates were analyzed for crude protein and amino acid profile. The liquid fraction after the protein extraction was analyzed for soluble protein concentration by Bio-Rad DC protein assay kit.

### 3.3. Hydrolysis of alfalfa proteins to peptides using protease enzymes

A 5% w/v (by dry weight) suspension of the dried acid precipitates containing alfalfa protein was prepared in pH 8.0 sodium phosphate buffer (0.1M). The slurry was incubated at 50°C, 150 rpm for 30 min, followed by the addition of Alcalase 2.4L (proteinase from *Bacillus licheniformis*) at the enzyme

dose of 5% w/w of dry solids. The protein hydrolysis was carried out at 60°C, 150 rpm for 4 hrs. The pH of hydrolysate was periodically checked and adjusted to 8.0 using 1M NaOH. Afterwards, the slurry was then heated to 85 °C for 10 min in boiling water to deactivate the enzymes. The liquid portion of the hydrolysate was separated and collected by centrifugation at 4500 rpm for 5 min and analyzed for peptide concentration using the Fisher Scientific Pierce Quantitative Colorimetric Peptide Assay Kit (Thermo scientific, MA).

### ***3.4. Antioxidative activity of alfalfa peptides***

Antioxidative properties of alfalfa leaf peptides obtained from the Section 3.3 were measured by reducing power assay described in Yen and Chen [21] and Xie et al. [7]. Reduced glutathione (GSH) was used as a standard for comparison. An aliquot of 2.5 mL Alfalfa leaf peptides and GSH solutions at different conc. (0 – 2.0 mg/mL) were prepared and mixed with 2.5 mL, 1% potassium ferricyanide solution. The mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL of 10% trichloroacetic acid. Then, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 mL) was mixed with 1.0 mL deionized water and 0.2 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm by using a spectrophotometer (Shimadzu UV-2600 Spectrophotometer, West Chicago, IL) after 10 min.

## **4. Project Objectives and Corresponding Results and Discussion**

### ***Objective 1: The effect of cell-wall degrading enzymes on protein extractability of alfalfa leaves***

The effect of cell-wall degrading enzymes on protein extractability of alfalfa leaves was measured to determine the optimal blend of enzymes (cellulases, hemicellulases, pectinases) that maximizes protein extraction yields. The selection of commercial enzyme preparations for disrupting cell wall matrix of alfalfa leaves was carefully based on their activities on the major structural polysaccharides of plant leaves, such as cellulose, hemicellulose, and other non-structural carbohydrates.

**Results:** A single enzyme blend of Viscozyme L at >10 mg/g dry alfalfa, a single blend of Pectinex Ultra SPL at >1.5 mg/g dry alfalfa, or a dual enzyme mixture comprising Viscozyme and Pectinex at minimum 6 mg/g dry alfalfa was determined as the optimum enzyme dose for protein extraction from alfalfa leaves. The enzyme-assisted, alkaline extraction of alfalfa proteins enhanced the protein extraction by a factor of 1.4–1.6 (equivalent to 65%–75% extraction yield with enzymes) at a low enzyme dose (< 6 mg/g dry alfalfa) as compared to the protein extraction without enzymes (47% without enzymes).

### ***Objective 2: The yields and quality of alfalfa leaf protein isolates and peptides***

The second goal was to measure the protein yields and amino acid profiles of protein extracted using enzymes and to compare the results to the non-enzymatic protein extraction method. The antioxidative property of peptides obtained from the alfalfa protein was also measured and presented in this section.

**Results:** The amino acid contents of extracted alfalfa leaf protein were comparable to those of isolated soybean protein reported in literature, except for glutamic acid and cysteine. The enzymatic hydrolysis of alfalfa leaf proteins using a protease (Alcalase) produced peptides with antioxidative activities as measured by the reducing power assay.

## 5. Results and Discussion

### 5.1. Dry vs. fresh leaves

The effect of drying alfalfa leaves on protein extractability was studied and presented in Figure 2. Fresh leaves used in this study were lyophilized to minimize the collapse of pores within the plant cell tissues, while the dry leaves were prepared from conventional air drying. The % protein extracted and solubilized from the control runs without enzymes was 41.0% and 46.8% for dry and fresh leaves, respectively. Fresh alfalfa leaves improved the protein yields only by 6% compared to dry leaves when enzymes were not used. The effect of drying leaves on enzymatic treatment was tested by adding a mixture of enzymes having equal volume of Cellic CTec 2, Viscozyme L and Pectinex Ultra SP-L at three different doses. The enzyme dose of 10 mg protein/g dry alfalfa enzyme, for example, resulted in about 0.9% v/v enzyme loading. Protein extractability was enhanced by roughly 30% for fresh leaves with respect to dried leaves with enzymes. At the lowest enzyme loading tested (10 mg/g dry alfalfa), the % protein extracted was 1.6 times greater for fresh leaves than for dried leaves. While the enzymes did not improve the protein extraction from dry leaves regardless of the amounts of enzymes added, they significantly increased the protein yields by 21-25% from fresh leaves compared to the control run. The results clearly show that drying leaves significantly reduced the protein extraction efficiency.

Typically, alfalfa is harvested and sun-cured in the field. Bales are dried further to less than 15% moisture for safe storage. While dried alfalfa leaves have the advantage of stable storage and ease of transportation, the naturally present pores within the leaf structure may shrink or even disappear as water molecules evaporate from the leaf material during the drying process. The shrinkage or disappearance of enzyme-accessible pores has a negative impact on the enzymatic saccharification of plant materials by limiting the mass transfer of enzymes to cell wall structure and the release of end-products of the hydrolysis [22, 23]. The size and distribution of internal and external pores of plant tissues also directly correlate with the surface area that is accessible to enzymes. The pores and surface area affect not only the yields but also the rate of enzymatic reactions.

The results indicate that drying of alfalfa leaves should be avoided as much as possible if the goal is to maximize the extraction yields of alfalfa proteins. However, processing fresh leaves can be problematic as it may involve additional costs related to handling and storage. Moisture control and pre-processing of leaves should be applied considering the processing and handling costs of alfalfa leaves, as well as the profitability of the final product. It should be noted that the extracted alfalfa proteins are considered value-added products, which have a higher market value than raw alfalfa.

Based on these results that indicate the unfavorable effect of drying of leaves on the protein extractability, all the subsequent experiments were conducted using the ground, fresh alfalfa leaves.

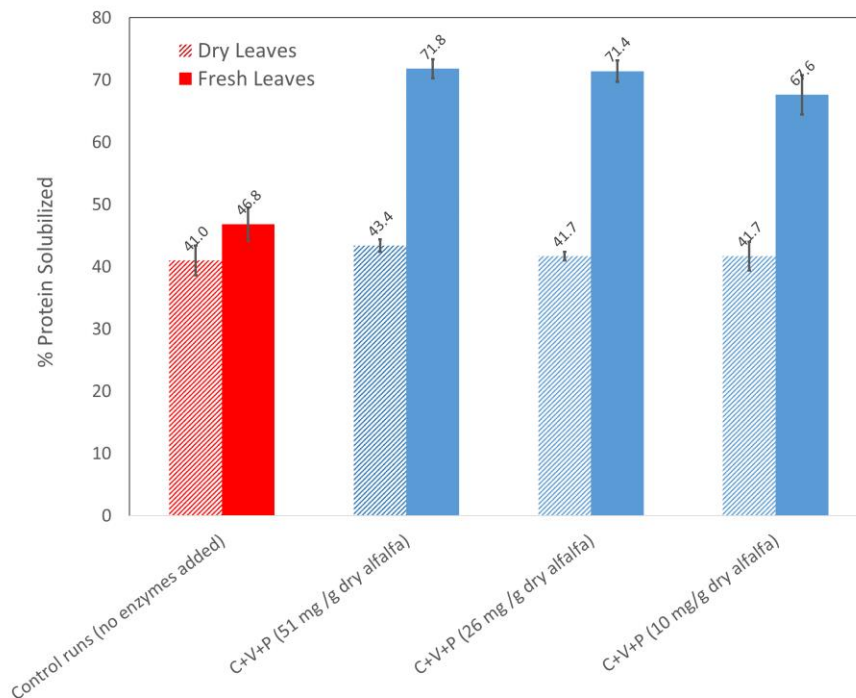


Figure 2. Effect of cell-wall degrading enzymes on protein extraction of dry vs. fresh alfalfa leaves. Enzymes added: C- Cellic CTec2, V- Viscozyme L, and P - Pectinex Ultra SP-L, combined at 1:1:1 volume ratio, 10 – 51 mg total enzyme proteins per g dry mass of alfalfa leaves. Enzyme treatment conditions: 6.4 % (w/v, by dry weight) ground alfalfa leaves in pH 5.0 sodium citrate buffer, incubated for 24 hrs at 50°C, 150 rpm, followed by adding 0.5M NaOH to adjust pH to 9.0 and incubation at 50°C, 150 rpm for 6 hrs.

### 5.2. Incubation time and its potential impact on a large-scale protein extraction

The duration of incubation in enzymatic treatments of biological materials is an important factor to consider due to its impact on the operational cost and the probability of contamination during the process. Many enzymatic reactions are carried out within the pH and temperature ranges that may promote undesirable microbial growth. The effect of incubation time on protein extraction was studied and the results are shown in Figure 3. The percentage of solubilized proteins from fresh alfalfa leaves reached 73.6% after 12 hrs of incubation using the three enzymes combined at 1:1:1 volume ratio, added at 51 mg enzyme protein per g dry alfalfa leaves. After 12 hrs of incubation, there was no further increase in protein extraction. The control runs (no enzymes) indicated that 50% protein extraction was reached between 2 and 4 hrs of incubation. The results suggest that the duration of incubation has minimal impact in the case of alkaline extraction of alfalfa proteins when no enzymes were used. On the other hand, enzyme-mediated extraction of alfalfa leaves requires at least 4-12 hrs of incubation to allow enzymes to act on the cell wall matrix of the leaves prior to alkaline treatment.

The incubation time may be further reduced with enzymes that are tuned to function at a more specific pH and temperature range and with a reactor or vessel designed for improved mixing characteristics in a large-scale process. The optimum conditions for the enzymes used in this study slightly vary. According to the product data sheet supplied by the manufacturer and literature, the optimum pH and temperature are pH 4.0–5.0 and 35–60°C for Pectinex Ultra SP-L [24], pH 3.3–5.5 and 25–55°C for Viscozyme L, and pH 5.0–5.5 and 45–50°C for Cellic CTec2. The enzymatic incubation conditions applied in this study were pH 5.0 and 50°C, which are within the optimal pH and temperature

ranges of each enzyme used. However, the incubation conditions could be further optimized and tuned for specific enzyme activities that are critical for improving the protein extraction from alfalfa leaves, rather than for the mixed blends. This may not only improve the protein extractability but also decrease the necessary incubation period of enzymatic treatment.

A limitation due to mixing characteristics and mass transfer, especially in high solids slurries, is another factor to consider in an enzymatic reaction. The concentration of solubilized protein in the supernatant, which subsequently goes through additional steps to be separated and recovered, is one of the key factors that affects the product recovery cost of alfalfa proteins. The more diluted the solubilized protein remains in the supernatant, the more costly the subsequent separation and purification process of the extracted protein will be. Therefore, the percent dry solids in the enzymatic reaction should be held at a level that is high enough to be beneficial to the separation and purification steps that follow the enzymatic reaction, but also at a level that is low enough to minimize the mass transfer limitation between the enzymes and substrates (i.e. plant cells). The negative impact of high % solids on the yields of enzymatic reaction of various cellulosic feedstock is well documented in various studies [11, 22, 25]. The % solids applied in this study was below 10%, which is considered a low to moderate solids loading in a typical industrial enzymatic reaction. At this solids level, the mass transfer and mixing limitations are considered insignificant. However, the impact of incubation time on the protein yields shown in Figure 2 might not be applicable in a large-scale operation, which needs to operate at a higher % solids to avoid the high costs in the downstream processing and purification of the extracted proteins. Further studies on identifying the key enzyme components in the enzyme mixtures, the impact of incubation conditions on individual enzyme activities, as well as on the impact of % solids on the protein extraction efficiency at different incubation periods are necessary to address these potential issues.

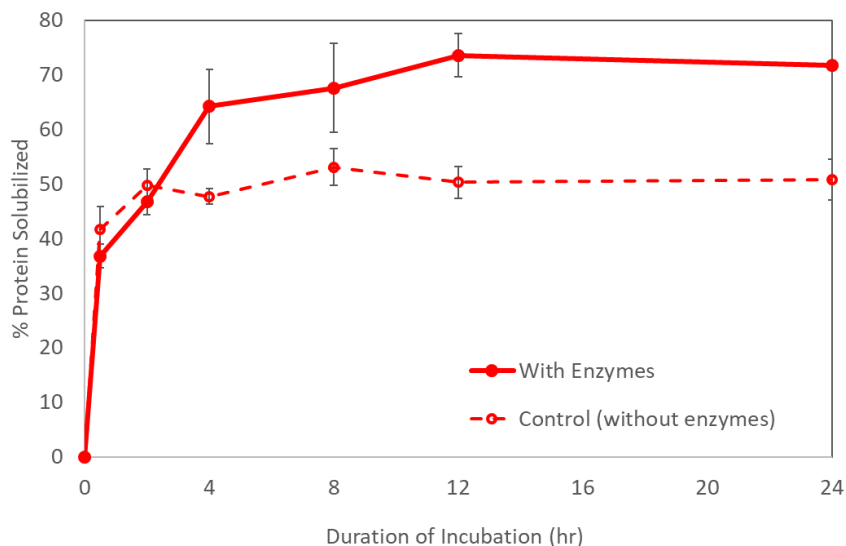


Figure 3. Effect of incubation time on protein extraction from fresh alfalfa leaves. Enzymes added: C- Cellic Ctec 2, V- Viscozyme L, and P - Pectinex Ultra SP-L, combined at 1:1:1 volume ratio, 51 mg total enzyme proteins per g dry mass of alfalfa leaves. Enzyme treatment conditions: 6.4 % (w/v, by dry weight) ground alfalfa leaves in pH 5.0 sodium citrate buffer, incubated for 0.5, 2, 4, 8, 12 and 24 hrs at 50°C, 150 rpm, followed by adding 0.5M NaOH to adjust pH to 9.0 and incubation at 50°C, 150 rpm for 6 hrs. Control: no enzymes were added.

### 5.3. Effect of individual enzyme on protein extraction from alfalfa leaves

The effect of each individual commercial enzyme blend on the yield of alfalfa leaf proteins was determined using the ground, fresh alfalfa leaves treated with a single enzyme added at different doses



(Figure 4). While 8 to 12 hrs of incubation was proven to be sufficient before the protein yield reaches a plateau using the mixture of all three enzymes (see Figure 3), the enzymatic treatment in this experiment was continued for 24 hrs to ensure the yield could reach a maximum for each individual enzyme blend even at a low dose. Figure 4 shows the % protein solubilized using a single enzyme preparation at two different enzyme loadings for each. Since commercial enzymes vary in the level of active enzymes in each formulation and contain various non-enzymatic components, the amounts of enzymes added in this study are given in the unit of mg protein per dry weight of alfalfa leaves. In addition, the cost of commercial enzyme blends correlates closely with the amount of enzymatic proteins they contain. Thus, studies on lignocellulosic conversion to fuels and other value-added chemicals using enzymes typically indicate enzyme doses in mg protein per unit dry mass of substrate [26, 27], rather than in the unit of w/w, v/v or w/v.

As indicated in Figure 4, Viscozyme L and Pectinex Ultra SP-L improved the protein extraction from alfalfa leaves, compared to a control run. Cellic CTec2 only marginally improved the yield by 8% even at the high enzyme dose of 139 mg protein/g dry alfalfa, which is equivalent to 7.1% v/v enzyme dose. The protein extraction with Cellic CTec2 at 39 mg/g dry alfalfa did not increase compared to the control. On the other hand, Viscozyme L enhanced the protein extractability by 12% and 20% at 5 and 10 mg/g dry alfalfa, respectively. Pectinex Ultra SP-L, which contains the least amount of mg enzyme protein per volume among the three enzyme blends used in this study, was the most effective in terms of increasing the protein extractability from the alfalfa leaves. At the enzyme dose less than 3 mg/g dry alfalfa the protein yield was close to 65-70%. The results suggest that Cellic CTec2 has no significant impact on the protein extraction from alfalfa leaves, while the other two enzyme blends enhance protein extraction by at least 12-23% even at a low enzyme loading (<10 mg/g dry alfalfa).

Cellic CTec2 is a cellulase complex enzyme (cellulases and  $\beta$ -glucosidases) which is developed to target cellulose fraction of lignocellulosic feedstock [28, 29]. Since it is not a significant source of hemicellulases and pectinases, it is often used with accessory enzymes that can breakdown the physical barrier of hemicellulose and pectin within plant cell walls [30, 31]. While Cellic CTec2 is an effective cellulolytic cocktail that can hydrolyze cellulose in plant cells, its effectiveness is limited to the hydrolysis of cellulose in pretreated or raw plant materials with low hemicellulose or lignin contents, unless it is supplemented with accessory enzymes such as hemicellulases and pectinases. Considering that cellulose is surrounded by other structural and non-structural polymers, such as hemicelluloses, pectin, and proteins which function as matrix and a physical barrier, Cellic CTec2 alone without auxiliary enzymes seems insufficient to disrupt the plant cell matrix of alfalfa leaves and to expose and release proteins from the leaves.

In contrast, Viscozyme L contains mainly  $\beta$ -glucanase, arabanase, cellulase, pentosanase, and xylanase activities [32]. Pectinex Ultra SP-L is a highly active enzyme preparation which is primarily composed of pectolytic activities including polygalacturonase, pectinlyase, pectinesterase, hemicellulase, cellulase, and amylase [33]. These enzyme blends are specifically designed for disintegrating plant cell walls of fruits and vegetables which are rich in pectin and other soluble, highly branched polysaccharides, rather than targeting cellulose. For example, the use of Viscozyme L on oat bran has shown that the enzymatic treatment enhances the protein extraction from oat bran [17]. Considering the more diverse enzyme activities targeting pectin and hemicellulose fractions of plant cell walls, these enzyme preparations were expected to perform better than Cellic CTec2 in terms of disintegrating and disrupting the cell wall matrix of alfalfa leaves.

Alfalfa leaves, compared to stems and other lignocellulosic materials, contain significantly higher proteins and less cellulose and lignin. In addition, the cell walls of alfalfa are known to contain a greater content of pectin than other grass cell wall materials [34]. Pectin, the major component of the cell-wall matrix, is a mixture of heterogeneous, branched, highly hydrated polysaccharides, with arabinose being the primary neutral sugar in alfalfa pectin with smaller amounts of galactose, rhamnose, glucose, mannose and fructose [35]. In the cell wall, the pectic polysaccharides form a gel-like network that is interlocked with the crosslinked cellulose microfibrils and hemicellulose. In addition, proteins in plant cell wall exist as being embedded and incorporated within cell matrix with other non-structural components. Enzymatic

hydrolysis of water-soluble arabinoxylan and pectin requires the enzyme activities that can cleave side chains or branches as well as the backbone of the polysaccharides. Figure 4 indicate that Viscozyme L and Pectinex Ultra SP-L contain the synergistic enzyme activities necessary for the depolymerizing and debranching the complex structure of pectin and hemicellulose of alfalfa leaves, thus enabling the extraction and solubilization of proteins embedded within plant cell wall.

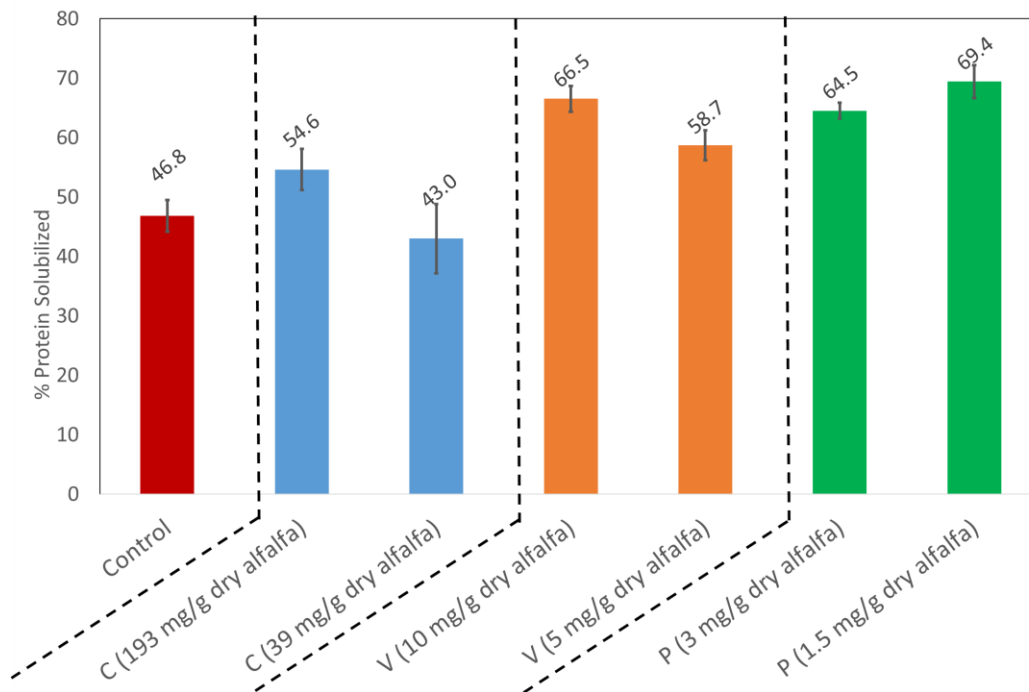


Figure 4. Effect of individual enzyme blend on protein extraction of fresh alfalfa leaves. Enzymes added: C- Cellic® Ctec 2 at 39 and 139 mg enzyme protein/g dry alfalfa (equivalent to 1.4 and 7.1 % v/v enzyme dose); V- Viscozyme L at 5 and 10 mg enzyme protein/g dry alfalfa (equivalent to 1.4 and 0.7% v/v enzyme dose); P - Pectinex Ultra SP-L at 3 and 1.5 mg enzyme protein/g dry alfalfa (equivalent to 1.4 and 0.7 v/v enzyme dose). Enzyme treatment conditions: 6.4 % (w/v, by dry weight) ground alfalfa leaves in pH 5.0 sodium citrate buffer, incubated for 24 hrs at 50°C, 150 rpm, followed by adding 0.5M NaOH to adjust pH to 9.0 and incubation at 50°C, 150 rpm for 6 hrs. Control: no enzymes were added.

The effect of multiple enzyme blends on the protein extraction is represented in Figure 5. Cellic CTec2, even in combination with other enzyme blends, had an insignificant impact on the protein extraction from alfalfa leaves. For example, the % solubilized protein for Viscozyme L and Pectinex Ultra SPL (V+P in Figure 5) blends mixed at a 1:1 ratio resulting in a 13 mg/g dry alfalfa dose was 74.7%, while the yield did not improve when the equal volume of Cellic CTec2 was added (see C+V+P at 51 mg/g dry alfalfa in Figure 5). The % solubilized protein with and without Cellic CTec2 was comparable to a lower enzyme dose as well (69.9% for V+P at 6 mg/g dry alfalfa vs. 71.4% C+V+P at 26 mg/g dry alfalfa). A dual enzyme mixture that contains Cellic CTec2 with either Viscozyme L or Pectinex Ultra SPL did not result in a yield improvement compared to a dual enzyme mixture comprising Viscozyme L and Pectinex Ultra SPL. These results show that cellulase activities are not critical in enhancing protein extractability from alfalfa leaves, even when supplemented with other auxiliary enzymes. If the goal was to hydrolyze the cellulose fraction of the leaves to obtain soluble sugars that can be utilized in the production of chemicals or fuels, adding cellulolytic activities augmented with hemicellulases would be critical in the process. However, the concentrations of soluble sugars (for

example, glucose, xylose, arabinose) that result from the hydrolysis of structural carbohydrates by the action of cellulolytic enzymes were not measured in this study as the analysis was beyond scope of this study.

Based on the results represented in Figure 4 and Figure 5, a single enzyme blend of Viscozyme L at >10 mg/g dry alfalfa, a single blend of Pectinex Ultra SPL at >1.5 mg/g dry alfalfa, or a dual enzyme mixture comprising Viscozyme and Pectinex at minimum 6 mg/g dry alfalfa was determined as the optimum enzyme dose for protein extraction from alfalfa leaves. The subsequent protein extraction for the analysis of amino acid profile and antioxidative activity of the extracted proteins was conducted using the dual enzyme blend of Viscozyme and Pectinex at 6 mg/g dry alfalfa loading.

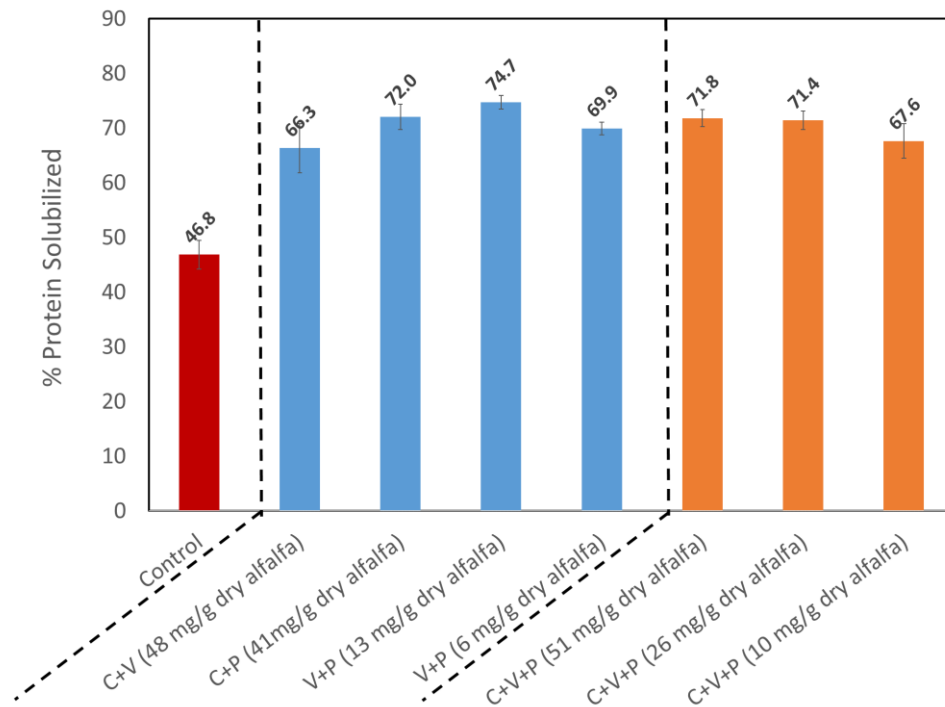


Figure 5. Effect of multiple enzyme blends on protein extraction of fresh alfalfa leaves. Enzymes added: Mixtures of enzymes combined at 1:1 (for dual enzyme mixtures) or 1:1:1 (for triple enzyme mixtures) v/v ratio. C- Cellic<sup>®</sup> Ctec 2; V- Viscozyme L; P - Pectinex Ultra SP-L. Enzyme treatment conditions: 6.4 % (w/v, by dry weight) ground alfalfa leaves in pH 5.0 sodium citrate buffer, incubated for 24 hrs at 50°C, 150 rpm, followed by adding 0.5M NaOH to adjust pH to 9.0 and incubation at 50°C, 150 rpm for 6 hrs. Control: no enzymes were added.

#### 5.4. Yields and amino acid profiles of alfalfa protein

The protein yields and amino acid profiles of protein extracted using enzymes were measured and compared to those from the non-enzymatic protein extraction method. To obtain a sufficient amount of alfalfa protein for the analysis of amino acid composition and antioxidative properties, a 2 L volume of 10% w/v (by dry weight) slurry of fresh, ground alfalfa leaves was treated with the 1:1 mixture of Viscozyme and Pectinex at 6 mg/g dry alfalfa enzyme dose for 8 hrs, followed by pH 9.0 alkaline treatment. The resulting liquid containing the extracted and solubilized alfalfa proteins was separated and acid treated to precipitate the soluble proteins.

*The mass balances of total solids and proteins of this process are shown in*

**Table 1.** *The initial mass of ground alfalfa leaves was 190 g (dry weight). The enzyme-assisted, alkaline extraction solubilized roughly a half of the initial dry mass. The subsequent acid coagulation precipitated 13% of the solubilized solids. The percentage of proteins solubilized by the enzyme-assisted alkaline extraction was about 63% (*

*Table 1), which was slightly lower than as shown in Figure 5 (70%). This is partially due to the shorter incubation period applied to the 2-L extraction run compared to the runs shown in Figure 5 (8 hrs vs. 24 hrs). The mass closure of proteins was over 100% (64.8 g/58.3 g=110%) due to some of the soluble proteins in the liquid being double counted as they remained in the liquid within the centrifuged solids. This means that the % protein content in the solids (28.6% as indicated in*

Table 1) was over-estimated by the crude protein analysis as the solids fraction also includes the solubilized proteins as well.

The solids fraction recovered through centrifugation after the protein extraction contained 28.6% proteins by dry wt. The precipitated solids upon acid treatment had a higher protein content of 35.6% protein (by dry wt.) than the raw, untreated alfalfa leaves (30.7%). In comparison, a control run without enzymes resulted in the acid precipitates with 26.4% protein (data not shown). Of the proteins solubilized in the extraction phase, however, only 11.5% (equivalent to 4.2 g out of 36.6 g) was precipitated and recovered, indicating that most of the solubilized proteins (88.5% of the total solubilized proteins in the liquid phase) could not be recovered through the low pH coagulation. The final protein yield was 7% based on the initial protein content.

The enzyme-assisted alkaline extraction of alfalfa proteins in this study resulted in a significantly higher % protein extraction (65%-75%, depending on the enzymes used) than the typical pressing method reported in the literature. Pressing of leaves typically achieve 20-40% protein extraction [36-39]. However, the final protein yield after extraction and coagulation was lower than the yields reported in the literature. Final protein yields from alfalfa leaves with respect to the initial protein content are typically in the range of 15-60% depending on the extraction and coagulation technique employed [3, 10]. The results imply that the acid coagulation is not an effective way to recover the extracted, soluble proteins with the use of enzymes. The conventional acid coagulation involves adjusting the pH of alfalfa juice to 3.5-4.0 by adding HCl, followed by the recovery of crude proteins by centrifugation or filtration [3, 4]. This method works because proteins lose their net charge at the isoelectric point (pI), causing them to aggregate and precipitate. If the surrounding pH is not close to the pI of a target protein, the efficiency of protein precipitation drops significantly. While the pH selected in this study to cause the protein precipitation was 3.5, which was within the typical range for alfalfa protein coagulation, the low recovery of the extracted soluble proteins through the acid coagulation suggests that the applied pH of 3.5 did not induce a sufficient protein coagulation. This could be due to the pI of the soluble proteins obtained through enzymes-assisted alkaline extraction not being close to pH 3.5 or the complex nature of the resulting extractives that contain other non-protein compounds.

Structural (cellulose, hemicellulose, lignin) and non-structural components (pectin, proteins, sugars) are present as a complex and intertwined network within plant cell wall, rather than completely being separate from each other [40]. For example, glycoproteins are cell wall proteins with carbohydrate chains covalently attached to amino acid side-chains, which may result in soluble proteins with sugar-based side-chains rather than pure proteins upon the enzymatic treatment. The use of cell-wall degrading enzymes with high pectin and hemicellulose degrading activities results in a complex mixture that comprises various hydrolysis compounds, including sugars, oligomers, and solubilized glycoproteins varying structures. The complex nature of the resulting extractives may affect the coagulation efficiency of the solubilized proteins. Indeed, the resulting acid precipitates contained non-protein components as

indicated by the measured crude protein content (35.6%). A different coagulation approach should be explored to improve the % protein recovery as well as the purity of the protein extract. Heat treatment, salting, polyelectrolyte-induced coagulation, organic solvent extraction, or membrane filtration are examples of the alternative approaches that could be explored to enhance the recovery of solubilized leaf proteins [4, 10].

Table 1. Mass balances of total solids and proteins obtained from alfalfa leaves using cell-wall degrading enzymes (ET: enzyme treatment; B: pH 9.0 alkaline treatment; A: pH 3.5 acid precipitation). Values are means of duplicate measurements.

Total Solids Mass Balance (dry weight)			
Initial	Solids recovered after ET + B	In liquid after ET+ B	Acid precipitated after ET + B + A
190 g	99 g	91* g (48% solubilized)	12 g (13% solids precipitated) <sup>†</sup>
Protein Content (% dry basis)			
30.7%	28.6%	n.a.	35.6%
Protein Mass Balance (dry weight)			
58.3 g	28.2 g	36.6 g (62.8% protein solubilized)	4.2 g (11.5% protein precipitated) <sup>†</sup>

\* determined by difference between initial – insolubilized

<sup>†</sup> % based on the mass solubilized after ET + B

The proteins recovered after acid coagulation were analyzed for the composition of amino acids (Figure 6). The protein obtained with enzyme-assisted, alkaline extraction and acid coagulation contained 30.1% total amino acids (by dry wt). In comparison, the protein obtained without the use of enzymes (control) contained 22.6% total amino acids. However, there was no statistically significant difference (p-value > 0.05) in amino acids compositions between the enzyme treated and control (data not shown). The contents of amino acids in alfalfa leaf protein in this study was comparable to those reported by Xie et al. [7]. The statistical significance of differences of the means could not be analyzed as the statistical information of the reported amino acids by Xie et al. was not provided. The leaf protein composition was also compared to that of isolated soy protein. Except for glutamic acid and cysteine, nearly all amino acids contents were close to those reported for isolated soybean protein. Several essential amino acids (Thr, Val, Ile, Leu, Phe, and Trp) were compared favorably to those in isolated soy protein. However, the statistical significance of the differences could not be assessed due to the same reason as explained above. These results suggest that alfalfa leaf protein has desirable nutritional quality and could be a source of protein-rich additives for livestock animals and potentially for human consumption.

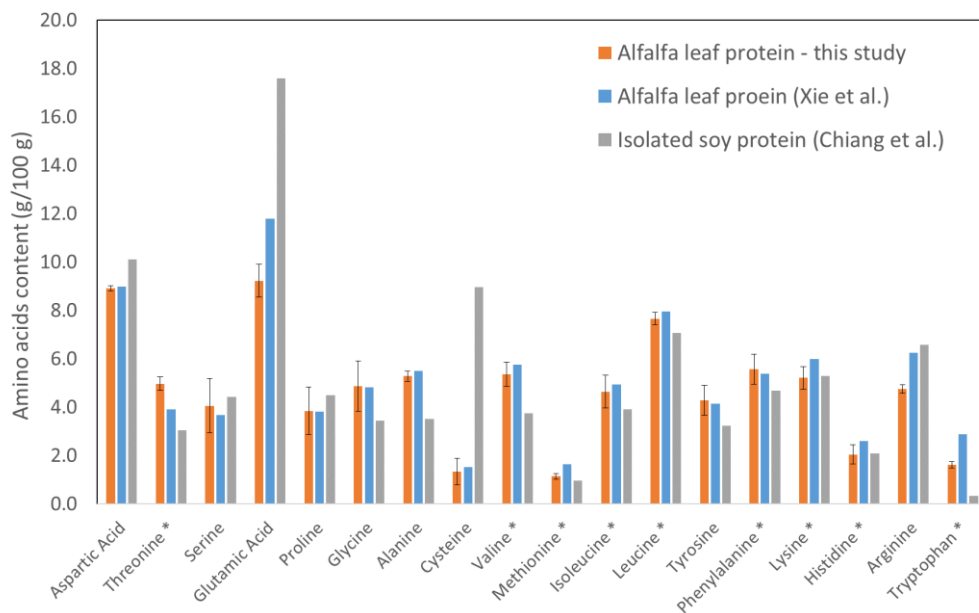


Figure 6. Amino acid compositions of alfalfa leaf proteins and isolated soy protein. \* : essential amino acids. Xie et al. [7], Chiang et al. [41]

### 5.5. Antioxidative properties of peptides obtained from alfalfa leaf proteins

The recovered alfalfa protein through acid coagulation was further hydrolyzed using a commercial protease (Alcalase 2.4L) to obtain peptides and assess their antioxidant activities. The analysis of hydrolysate for peptide concentration indicated that 87% of the alfalfa protein in acid precipitate was hydrolyzed to peptides. The resulting hydrolysate contained 16 g/L peptides when 5% w/v slurry of acid precipitates was hydrolyzed with Alcalase. The antioxidant activities of peptides were measured by reducing power assay, which measures the antioxidant's ability to donate electrons or hydrogen atoms [42]. Reduced glutathione (GSH) was used as a reference material for comparison.

The measured reducing power of alfalfa peptides is compared to that of GSH in Figure 7 at different concentrations. The higher absorbance at 700 nm indicates the greater reducing power [7]. At all concentrations tested, alfalfa peptides exhibited lower reducing power than reduced glutathione. It appears that roughly two times higher concentration of alfalfa peptides than that of GSH exhibits an equivalent level of reducing power to GSH. The results are also comparable to those previously reported by Xie et al. [7], which showed that reducing power of alfalfa peptides were close to that of GSH at three times the concentration of GSH. It should be noted that the measured antioxidative property could be due to not only peptides but also other compounds in the sample. The acid precipitate contains 35.6 % protein, meaning 64.4% of its dry matter is non-protein constituents. Some of the non-protein constituents, such as oligomeric sugars and phenolic compounds, might have contributed to the measured reducing power assay as well. In addition, it is noteworthy that the presence of some free amino acids, such as Trp, Tyr, Met, Cys, His, Phe and Pro, had been reported to contribute positively to the antioxidant activities of peptides [43]. The presence of these antioxidative free amino acids in alfalfa leaf protein (see Figure 6) may have also promoted the antioxidant activities of alfalfa protein hydrolysate.

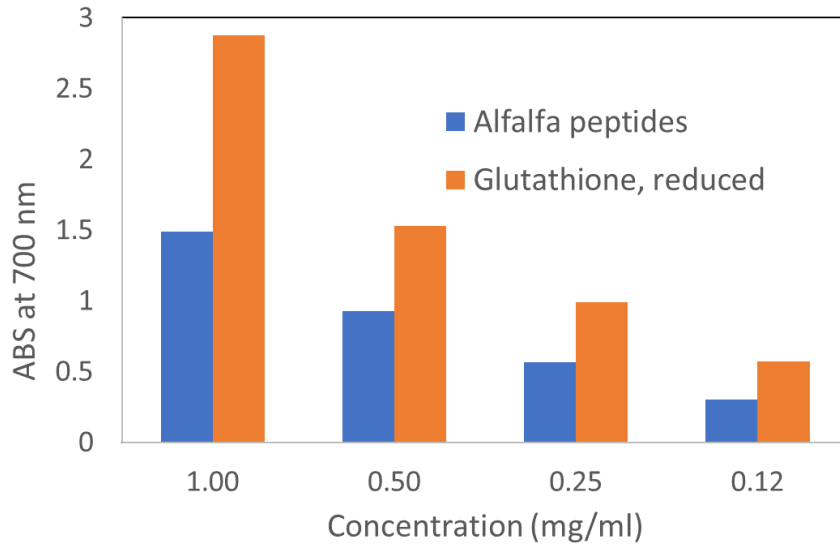


Figure 7. Reducing power of alfalfa peptides vs. reduced glutathione. Values are the means of duplicate measurements.

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Alfalfa, Protein, Leaf protein, Cell-wall degrading enzymes, Cellulases, Pectinases, Protein extraction, Antioxidant