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Isolation of Free Amino Acids via Enzymatic Hydrolysis of Tobacco-Derived F1 Protein *

by

Mehdi Ashraf-Khorassani [1], *William M. Coleman III* [2], *Michael F. Dube* [3], *and Larry T. Taylor* [1]

[1] *Department of Chemistry, Virginia Tech, Blacksburg, VA 24061, USA*
[2] *iii Consulting, 210 Jessamine Street, Conway, SC 29526, USA*
[3] *R.J. Reynolds Tobacco Company, 950 Reynolds Blvd, Winston Salem, NC 27105, USA*

SUMMARY

Free amino acids have been isolated via optimized enzymatic hydrolysis of F1 tobacco protein using two cationic resins (Amberlite IR120 and Dowex MAC-2). Optimized enzymatic conversions of the protein as a result of systematic variations in conditions (e.g., time, temperature, pH, enzyme type, enzyme concentration, anaerobic/aerobic environments, and protein concentration) employing commercially available enzymes, were consistently higher than 50% with qualitative amino acid arrays that were consistent with the known composition of tobacco F1 protein. Amberlite IR120 was shown to have a much higher efficiency and capacity for isolation of amino acids from standard solutions and from hydrolysate when compared with the results using Dowex MAC-2. Two columns packed with conditioned Amberlite IR120 (120 × 10 mm,12–15 g resin) and (200 × 25.4 mm, 60–65 g resin) were used to isolate two batches (2.5–3.0 mg and 13–15 mg) of free amino acids, respectively. A relatively inexpensive analytical methodology was developed for rapid analysis of the free amino acids contained within the enzyme hydrolysate. Commercially available enzymes, when employed in optimized reaction conditions, are very effective for enzymatic conversion of tobacco F1 protein to free amino acids. [Beitr. Tabakforsch. Int. 28 (2018) 179–190]

ZUSAMMENFASSUNG

Freie Aminosäuren wurden unter Einsatz von zwei kationischen Harzen (Amberlite IR120 und Dowex MAC-2) mittels optimierter enzymatischer Hydrolyse des Tabakproteins F1 isoliert. Die optimierten enzymatischen Umwandlungsraten des Proteins nach einer systematischen Variation der Bedingungen mithilfe handelsüblicher Enzyme (z.B. Zeit, Temperatur, pH-Wert, Enzymtyp, Enzymkonzentration, anaerobe/aerobe Umgebung und Proteinkonzentration) waren konstant höher als 50%, und die qualitativen Aminosäurearrays stimmten mit der bekannten Zusammensetzung des Tabakproteins F1 überein. Es zeigte sich, dass Amberlite IR120, verglichen mit den beim Einsatz von Dowex MAC-2 erzielten Ergebnissen, über einen deutlich höheren Wirkungsgrad und eine viel stärkere Kapazität zur Isolierung von Aminosäuren aus Standardlösungen und aus Hydrolysat verfügt. Zur Isolierung von jeweils zwei Batches freier Aminosäuren (2,5–3,0 mg und 13–15 mg) wurden zwei Säulen mit konditioniertem Amberlite IR120 (120 × 10 mm, 12–15 g Harz und 200 × 25.4 mm, 60–65 g Harz) befüllt. Es wurde eine relativ kostengünstige analytische Methodik zur Schnellanalyse der im Enzymhydrolysat enthaltenen freien Aminosäuren entwickelt. Unter optimierten Reaktionsbedingungen sind handelsübliche Enzyme bei der enzymatischen Umwandlung des Tabakproteins F1 in

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freie Aminosäuren sehr effektiv. [Beitr. Tabakforsch. Int. 28 (2018) 179–190]

RESUME

Des acides aminés libres furent isolés grâce à une hydrolyse par voie enzymatique optimisée de la protéine de tabac F1 et l'utilisation, en l'occurrence, de deux résines cationiques (Amberlite IR120 et Dowex MAC-2). Les conversions enzymatiques optimisées de la protéine résultant des variations systémiques des conditions (par exemple, le temps, la température, le pH, le type d'enzyme, la concentration de l'enzyme, les environnements anaérobies/aérobies et la concentration de la protéine) utilisant les enzymes disponibles dans le commerce furent constamment supérieures à 50% et les gammes qualitatives d'acides aminés concordèrent avec la composition connue de la protéine de tabac F1. Il apparut que l'Amberlite IR120 présentait une efficacité et une capacité bien plus élevées à isoler les acides aminés à partir de solutions normalisées et à partir d'hydrolysats comparativement aux résultats obtenus grâce au Dowex MAC-2. Deux colonnes remplies d'Amberlite IR120 conditionnée (120 × 10 mm, 12–15 g de résine et 200 × 25,4 mm, 60–65 g de résine) furent utilisées pour isoler deux lots respectifs d'acides aminés (2,5–3,0 mg et 13–15 mg). Une méthodologie analytique relativement peu coûteuse fut mise au point pour une analyse rapide des acides animés libres contenus dans l'hydrolysat enzymatique. Les enzymes disponibles dans le commerce, lorsqu'elles sont employées dans des conditions de réaction optimisées, s'avèrent très efficaces pour la conversion enzymatique de la protéine de tabac F1 en acides aminés libres. [Beitr. Tabakforsch. Int. 28 (2018) 179–190]

Abbreviations	
AA	Amino acids
ALS	Automated Liquid Sampler
DAD	Diode array detector
DI H_2O	Deionized water
HPLC	High Performance Liquid Chromatography
ID	Internal diameter
ISTD	Internal standard
OPA	o-Phthalaldehyde
RSD	Relative standard deviation
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oxygenase

INTRODUCTION

Amino acids are well known for their ability to react with sugars in the Maillard reaction to produce Amadori compounds as well as a wide array of flavor materials, among them pyrazines which possess for the most part positive sensory attributes and acceptable chemical characteristics (1–12). Furthermore, in selected cases, the structure of the amino acid has been clearly shown to dictate the structure of the resulting pyrazines and in addition, the structure of the pyrazines dictates its sensory character (8, 9, 13). In addition, tobacco F1 protein which is known for its diverse array of amino acids, often compared well alongside profiles known for soy and egg (13). Release of protein-bound amino acids into their free form would afford an opportunity to react liberated free amino acids with selected sugars in order to prepare an array(s) of unique pyrazines (3, 6, 7, 12). In general the F1 protein in tobacco is ribulose-1,5-biphosphate carboxylase/oxygenase and is commonly referred to with the abbreviation RuBisCO. Some amino acids that participate in Maillard reactions are threonine, valine, alanine, leucine and isoleucine. The latter four of these amino acids are known for their capability for producing Strecker aldehydes which participate in the formation of pyrazines. Usually pyrazines thus produced have alkyl sidechains bearing structures similar to those present in the free amino acid alkyl chains. Thus, these free amino acids from F1 protein can be envisioned to serve as nitrogen sources in reactions designed to prepare arrays of pyrazines. In order to determine which amino acids are present in a protein hydrolysate, a suitable technique to isolate, separate, quantify, and identify as many of the free amino acids as possible was very important. Conventional methods for free amino acid analyses are readily available, but often they require special and relatively expensive instrumentation which are sold by different manufacturers (e.g., Agilent, Hitachi, etc.). A more facile, rapid, and less expensive approach for the quantitative determination of free amino acids would thus be of great benefit. General conditions for effective enzymatic hydrolysis of protein have been well described employing commercially available enzymes (14–16). However, most hydrolysis procedures require optimization conditions specific to the protein under investigation. Likewise, general optimized cationic column chromatographic approaches focused on isolation of free-liberated amino acids have been documented as well (17, 18) but they require optimization based on the array and concentration of free amino acids present in an hydrolysate.

In this study, two cationic resins have been examined for their potential to effectively isolate the amino acids and to minimize the volume of hydrolyzed protein solution required. Results related to a series of experiments directed at optimizing reaction conditions (e.g., time, temperature, pH, enzyme type, enzyme concentration, anaerobic environment, and protein concentration) for the quantitative enzymatic hydrolysis of tobacco derived F1 protein coupled with methods directed at quantifying and isolating the free amino acids from the hydrolyzed protein will be described.

EXPERIMENTAL

Materials

Amberlite IR120 and Dowex MAC-2, ammonium hydroxide (28–30%), amino acid standards, o-phthalaldehyde (OPA), borate buffer, and concentrated HCl were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tobacco-derived F1 protein was obtained from R.J. Reynolds Tobacco Co. Borate buffer solution (0.4 N) was prepared by mixing 2.48 g of boric acid and 2.98 g potassium chloride in 100 mL of deionized (DI) water wherein pH was adjusted to 10.2. Both Maxipro NPU and FPC enzymes were obtained from DSM (Exton, PA, USA).

Instrumentation

All HPLC/UV analyses were performed using an Agilent (Santa Clara, CA, USA) 1100 series HPLC equipped with a diode array detector (DAD), a well plate automated liquid sampler (ALS), a column heater, and quaternary pump. Separations were obtained using a Zorbax SB-C_{18} (150 × 3.0 mm, 5 µm), and an Eclipse XDB C_{18} (150 × 4.6 mm, 5 µm) columns from Agilent (Figure 1). All amino acids (AA) were monitored using a DAD detector at 338 nm after online derivatization with o-phthalaldehyde. Mobile phase A contained 40 mM Na_2HPO_4 (5.5 g of NaH_2PO_4, monohydrate + 1 L of water, adjusted to pH 7.8 with 10N NaOH solution). The following mobile phase gradient program was used to obtain the separation of amino acids:

Time (min)	A (%)	B (%)	Flow (mL/min) (SB-C_{18})	Flow (mL/min) (XDB-C_{18})
0	95	5	0.8	1.0
2	90	10	0.8	1.0
19	57	43	0.8	1.0
19.1	0	100	0.8	1.0
22	0	100	0.8	1.0
22.1	95	5	0.8	1.0
24	95	5	0.8	1.0

The diluent solution was a mixture of 100 mL of Mobile phase A + 0.25 mL of H_3PO_4.

Automated Liquid Sampler (ALS) on-line derivatization

The following program was used for on-line derivatization:

- Draw 2.5 µL of borate buffer
- draw 1 µL of sample
- mix 3 µL "in air", 10 times, wait 0.5 min
- draw 0.5 µL of OPA (o-phthalaldehyde)
- mix 4.4 µL "in air", 10 times
- draw 32 µL of diluent solution
- mix 18 µL "in air", 10 times
- inject.

Amino acid standards and internal standard

A mixture of 15 amino acids was prepared at a concentration of 10 nmole/µL. This stock solution was used to prepare 5 standards with concentrations ranging from 100–1000 nmole/µL. For all analyses 50 µL of internal standard trans-4-(aminoethyl)-cyclohexane carboxylic acid (10 nmole/µL) was added to each standard and sample prior to analysis. For all analyses of hydrolyzed F1 protein, 950 µL of hydrolyzed solution was spiked with 50 µL of internal standard (ISTD) to adjust the volume to 1 mL. Figure 2 shows the calibration curves for each amino acid in the mixture of standards. Glutamine and proline were not measured. The results of the qualitative and quantitative amino acid analyses employing this approach were in general agreement with similar findings that employed a significantly different analytical approach (19).

Resin preparation, loading, washing, and elution of the amino acids

Prior to loading the resin with hydrolyzed protein, bulk resin was washed a couple of times with deionized water until the solution was clear. Then the resin was washed two times with 1N HCl and each time enough 1N HCl was used to cover all the resin. In each wash, resin was stirred very gently in the solution for approximately 20–25 min. After washing with 1N HCl, the filtrate was washed 3–4 times with DI water. Again, the water level in each wash covered all the resin. Finally, the washed resin was loaded into the column. Two columns were prepared. The first column packed with resin had a length of 120 mm and internal diameter (ID) of 10 mm and the second column had a length of 200 mm and ID of 25.4 mm. For the 10 mm column, prior to loading with sample, the column was washed with 20 mL of DI H_2O. Then the sample which was usually 1 mL of hydrolyzed F1 protein (or 50–100 µL of standard amino acids) was mixed with 12–15 mL of 0.1N HCl and was loaded onto the column. The solvent was eluted from the column at a rate of 1–2 mL/min. Then the column was washed twice with 15 mL of DI H_2O. In the first wash, DI H_2O was eluted at a rate of 2–3 mL/min and in the second wash it was eluted with the maximum flow rate. Finally, after the second wash, amino acids were eluted from the column using 15 mL of 7N NH_4OH at a rate of 1–2 mL/min. In all steps, the level of solvent at the top of the column did not go below the resin level. After elution of amino acids from the column, the eluted solvent was evaporated via rotary evaporator, and solid material was re-dissolved in 2 mL of 0.1N HCl. The resulting solution was analyzed later via HPLC.

F1 Protein optimization parameters

In order to optimize the free amino acid yield from the F1 protein hydrolysis, initial hydrolysis manufacturer conditions were chosen: Temperature 50 °C, pH 7.0, hydrolysis time 20 h, 1 g of F1 protein mixed with 100 mL of H_2O and enzymes were added 5% w/w (Enzyme/F1protein).
At each step of optimization, one reaction condition was varied while other conditions were kept constant. For example, the pH of the solution was adjusted to 6.3, 7.0, and 7.8 using 1N HCl or 1N NaOH, while other parameters such as time, temperature, enzyme concentration and F1 protein concentration in H_2O were kept the same as in the original parameters (20 h, 50 °C, enzyme/F1 protein 5% (w/w) 1 g F1 protein in 100 mL H_2O). The following parameters were optimized:

- pH: 6.3, 7.0, and 7.8
- Temperature: 45, 50, and 58 °C
- Hydrolysis time: 1, 4, 12, 18, 20, 24, 36, and 48 h
- Type of enzyme: Maxipro NPU and FPC
- Enzyme concentration: 2.5, 5.0, 10, and 20% (w/w) enzyme/F1 protein
- Concentration of F1 protein: 1 g in 200 mL H_2O, 1 g in 100 mL H_2O, and 1 g in 50 mL H_2O
- Aerobic *vs*. anaerobic environment.

After hydrolysis, the suspension was filtered using Whatman #1 filter paper prior to analysis.

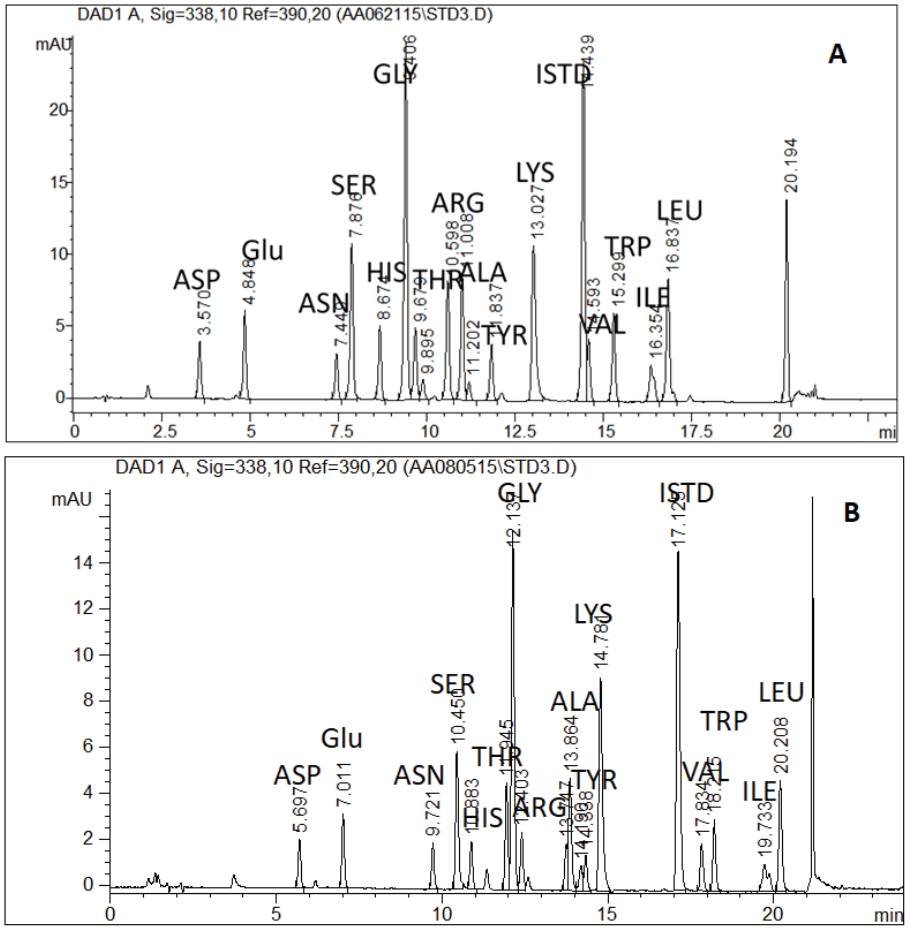

Figure 1. HPLC separation of amino acids after pre-derivatization using Zorbax SB-C_{18} (150 × 3.0 mm, 5 µm) column (A) and Eclips XDB-C_{18} (150 × 4.6 mm, 5 µm) column (B) from Agilent. See EXPERIMENTAL for chromatography conditions.

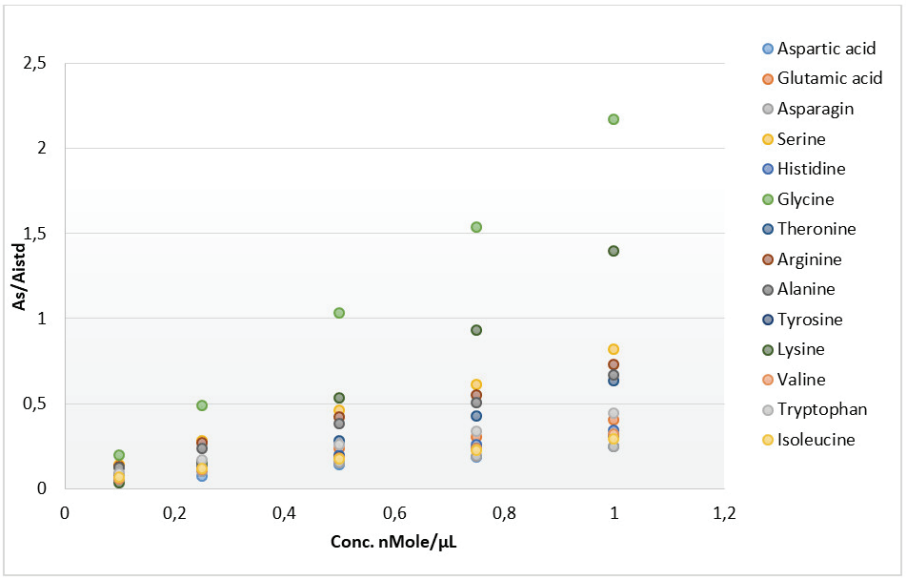

Figure 2. Calibration curves for free amino acids.

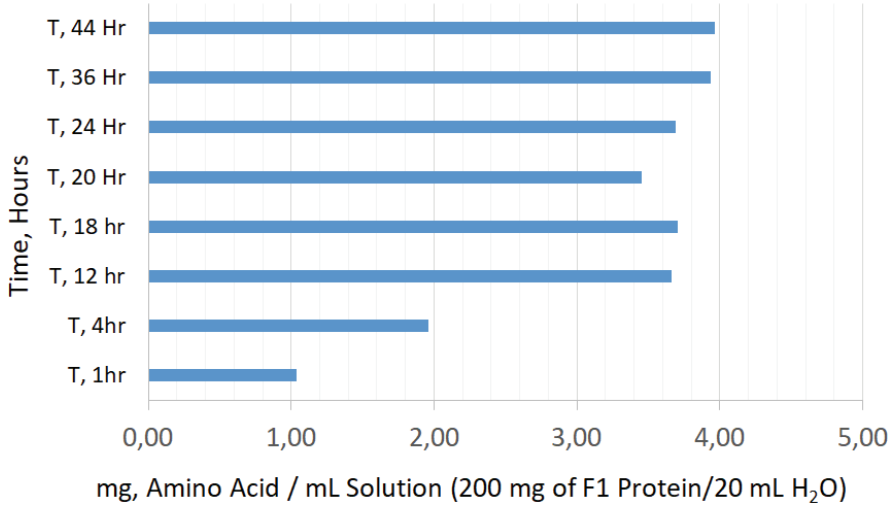

Figure 3. Impact of hydrolysis reaction time on free amino acid yields.

RESULTS AND DISCUSSION

Optimization parameters for hydrolysis of F1 protein

In order to optimize the hydrolysis of F1 protein, suggestions as to the types of enzymes to use and the initial hydrolysis conditions were solicited from several manufacturers. After establishing with the manufactures that the desired result was maximum free amino acids content due to enzymatic hydrolysis of tobacco F1 protein, the manufactures provided their specific recommendations. Based on these findings, the following two enzymes, Maxipro NPU (Endo Neutral Protease) and Maxipro FPC (Endo/Exo Fungal Protease), were selected. Thus, initially following specific manufacturer recommendations, 1 g of F1 protein was mixed with 100 mL of H_2O, 50 mg of Maxipro NPU, and 50 mg of Maxipro FPC. The mixture pH was adjusted to 7.0 and it was incubated at 50 °C for 20 h, resulting in 37–39% protein hydrolysis. In order to possibly increase this enzymatic hydrolysis conversion to at least 50%, different reaction parameters were examined to optimize free amino acid yields.

Effect of pH on hydrolysis of F1 protein

Three solutions in triplicate were prepared with 200 mg of F1 protein (F1) + 20 mL of H_2O + 10 mg Maxipro NPU and 10 mg Maxipro FP. For the three solutions, pH was sequentially adjusted to 6.3, 7.0, and 7.8 using 1M NaOH or 1M HCl. All three solutions were shaken at 50 °C for 20 h. After 20 h, all samples were heated at 85 °C for 10 min to deactivate the enzyme. Then, 950 µL of each solution was mixed with 50 µL of ISTD, and the resulting solution was filtered and analyzed via HPLC. Figure 3 shows a typical chromatogram of hydrolyzed F1 protein when the pH was adjusted to 7.0. Table 1 shows the calculated concentration of each amino acid obtained via hydrolysis of F1 protein at the different pH. Hydrolysis yields increased from 31.7% to 35.1% as the initial pH values increased from 6.3 to 7.8. Of note was the final pH values of the hydrolysis reactions, all of which decreased significantly, as follows: from 6.3 to 5.95, from 7.0 to 6.2, and from 7.8 to 6.75.

Table 1. Effect of pH on free amino acid levels after hydrolysis of F1 protein. Reaction conditions: 200 mg of F1 + 20 mL of H_2O, temperature 50 °C, 10 mg Maxipro NPU and 10 mg Maxipro FPC, hydrolysis time 20 h.

Initial pH	6.3	7.0	7.8
Yield amino acid (µg/mL)			
Aspartic acid	129	130	137
Glutamic acid	173	176	183
Asparagine	94	107	119
Serine	110	127	148
Histidine	78	81	78
Glycine	95	100	114
Threonine	189	196	197
Arginine	213	226	235
Alanine	230	257	297
Tyrosine	391	343	352
Lysine	355	342	331
Valine	378	417	461
Tryptophan	49	58	73
Isoleucine	315	325	358
Leucine	374	396	423
Total weight (mg/mL)	3.17 (1.0)*	3.28 (1.1)*	3.51 (1.0)*

* % RSD

Effect of temperature on hydrolysis of F1 protein

Three temperatures (45, 50, and 60 °C) were tested to determine hydrolysis yield. Each experiment was performed in triplicate. Parameters for hydrolysis in these experiments were similar to the preliminary conditions (200 mg of F1, 20 mL of H_2O, 10 mg of Maxipro NPU and 10 mg of Maxipro FPC, pH adjusted to 7.0 and time was set to 20 h). Percent yields of amino acids from hydrolysis of F1 protein at 45 °C and 50 °C were 35.7 and 34.5%, respectively. The percent yield at 60 °C was, however, much lower. This may be due to a decrease in enzyme activity at higher temperatures (Table 2). Percent RSD was less than 5% for triplicate runs at each temperature.

Table 2. Effect of temperature on hydrolysis of F1 protein. Reaction conditions: 200 mg of F1 + 20 mL of H_2O, pH 7.0, 10 mg Maxipro NPU and 10 mg Maxipro FPC, hydrolysis time 20 h.

Reaction	45 C°	50 °C	60 °C
Yield amino acid (µg/mL)			
Aspartic acid	190.4	182.8	96.7
Glutamic acid	309.8	190.4	150.2
Asparagine	137.6	123.1	102.6
Serine	169.8	135.8	86.9
Histidine	81.2	112.0	76.1
Glycine	139.4	90.6	70.7
Threonine	244.9	206.1	155.9
Arginine	59.4	222.9	234.1
Alanine	330.2	281.8	195.5
Tyrosine	142.0	323.0	268.6
Lysine	372.7	305.9	322.5
Valine	501.7	456.9	387.1
Tryptophan	88.6	78.7	62.4
Isoleucine	346.1	322.0	288.5
Leucine	457.8	422.3	393.6
Total weight (mg/mL)	3.57 (3.5)*	3.45 (2.8)*	2.98 (4.8)*

* % RSD

Table 3. Effect of enzyme concentration on hydrolysis of F1 protein. Reaction conditions: 200 mg of F1 + 20 mL of H_2O, pH 7.0, temperature 50 °C, hydrolysis time 20 h, enzyme mixture: Maxipro NPU and Maxipro FPC.

Reaction temperature (mg)	5	10	20	40
Yield amino acid (µg/mL)				
Aspartic acid	108.8	182.8	190.1	254.8
Glutamic acid	158.1	190.4	246.7	266.0
Asparagine	85.5	123.1	155.2	173.7
Serine	99.4	135.8	154.8	198.7
Histidine	69.3	112.0	103.7	108.8
Glycine	80.0	90.6	110.4	142.7
Threonine	164.3	206.1	238.8	262.6
Arginine	192.2	222.9	272.5	302.5
Alanine	216.9	281.8	292.8	345.8
Tyrosine	261.3	323.0	373.0	419.5
Lysine	300.1	305.9	380.0	419.9
Valine	388.0	456.9	503.0	515.5
Tryptophan	62.2	78.7	84.0	101.9
Isoleucine	262.9	322.0	354.5	366.4
Leucine	367.3	422.3	469.3	494.0
Total weight (mg/mL)	2.82	3.45	3.93	4.37

Effect of enzyme concentration and type of F1 hydrolysis with F1 protein

The effect of enzyme concentration on hydrolysis of F1 protein was investigated. The concentration of enzyme in the initial experiment decreased by 5% (5 mg) and in another experiment increased by 100% (20 mg) and 300% (40 mg). Results showed that decreasing the enzyme concentration by 50% decreased the yield of amino acids by more than 18%; while, increasing the enzyme concentration from 10 to 20 and 40 mg in the hydrolysis process increased the yield of amino acids by 14 and 27%, respectively. The results did not show that increasing enzyme concentration led to a corresponding decrease in percent yield of any specific amino acid (Table 3).

In addition, the effect of an individual enzyme and mix of both enzymes on hydrolysis of F1 protein was studied. In this part of the study, when only one enzyme was used, instead of using an equal mass of both enzymes for the reaction, only one enzyme (at double concentration) was used in different reactions to determine the percent yield of hydrolysis. Results showed that Maxipro FPC enzyme was 3 times more effective than Maxipro NPU in the hydrolysis of F1 protein. No research was conducted to determine if enzymes were selective for production of a specific amino acid (Table 4).

Table 4. Effect of enzyme type on hydrolysis of F1 protein. Reaction conditions: 200 mg of F1 + 20 mL of H_2O, pH 7.0, temperature 50 °C, hydrolysis time 20 h. Total mass of enzymes for each reaction: 20 mg.

Protein	FPC	NPU	Mix of both
Yield amino acid (µg/mL)			
Aspartic acid	178.4	39.4	182.8
Glutamic acid	247.7	63.1	190.4
Asparagine	129.2	0.0	123.1
Serine	142.0	23.7	135.8
Histidine	89.8	0.0	112.0
Glycine	109.6	48.9	90.6
Threonine	208.8	59.7	206.1
Arginine	243.5	98.7	222.9
Alanine	271.2	125.5	281.8
Tyrosine	331.9	124.9	323.0
Lysine	349.7	12.6	305.9
Valine	436.8	161.5	456.9
Tryptophan	74.6	0.0	78.7
Isoleucine	287.9	112.1	322.0
Leucine	410.3	199.0	422.3
Total weight (mg/mL)	3.51	1.13	3.45

Duplicate reactions were conducted for each group.

Effect of F1 protein concentration on hydrolysis

Since the volume of the hydrolysis solution had to be decreased after each hydrolysis step, it was important to determine if the F1 protein concentration had any effect on hydrolysis. For this reason, hydrolysis of F1 protein was obtained at three different concentrations. This was carried out by decreasing the volume of H_2O in each experiment while keeping other parameters unchanged. The initial concentration of F1 protein was 1% in water. Subsequent experiments were performed when the mass of F1 protein in H_2O was at the 0.5%- and 2.0%-level (200 mg of F1 protein in 40 mL or 10 mL H_2O). Results showed that by decreasing the F1 protein concentration, less protein was hydrolyzed. However, when the H_2O volume was decreased by half of the original reaction volume, more protein was hydrolyzed (Table 5).

Table 5. Effect of protein concentration on hydrolysis of F1 protein. Reaction conditions: 200 mg of F1 protein in 10, 20, or 40 mL H_2O, pH 7.0, temperature 50 °C, hydrolysis time 20 h. Total mass of enzymes for each reaction: 20 mg.

Volume of water (mL)	10	20	40
Yield amino acid (µg/mL)			
Aspartic acid	194.4	182.2	127.0
Glutamic acid	230.2	190.4	154.9
Asparagine	133.0	123.1	80.4
Serine	145.6	135.8	103.5
Histidine	89.5	112.0	57.1
Glycine	96.9	90.6	88.8
Threonine	245.3	206.1	167.6
Arginine	232.7	222.9	219.7
Alanine	289.2	281.8	240.7
Tyrosine	341.9	323.0	292.4
Lysine	343.5	305.9	360.7
Valine	470.8	456.9	420.5
Tryptophan	80.0	78.7	51.8
Isoleucine	303.6	322.0	256.4
Leucine	446.2	422.3	401.5
Total weight (mg/mL)	3.64	3.45	3.03

Duplicate reactions were conducted for each group.

Effect of reaction time on F1 protein hydrolysis

It was important to determine how much time is required to optimize the hydrolysis reaction, that is, the free amino acid yield. For this purpose, two reactions using the initial conditions stated previously were started simultaneously, and at different times, samples were taken and analyzed for percent hydrolysis. Samples were taken at 1, 4, 12, 18, 20, 24, 36, and 44 h. Table 6 shows the average calculated concentration of each AA obtained from hydrolysis of F1 protein at different times. Results indicated that as time increased, more F1 protein was hydrolyzed. The data showed that only about 8% increase in hydrolysis was observed when the hydrolysis time increased from 12 to 44 h. Figure 3 shows the results of Table 6.

Effect of aerobic/anaerobic environment for F1 protein hydrolysis

Next, hydrolysis of F1 protein was performed in the absence and the presence of air, Table 7. No difference in percent yield of amino acids with and without the presence of air was found. This was within the margin of error of both analytical method and process.

F1 protein hydrolysis using optimum conditions

After determining the optimized conditions, additional optimization experiments were performed by adjusting more than one parameter at the same time. For this purpose, triplicate hydrolysis reactions of F1 protein were performed using two different conditions. For the first set of reactions, the pH and temperature were set to 7.0 and 50 °C, respectively. However, in the second set of reactions, the pH and temperature were set to 7.8 and 47 °C (Table 8). Other reaction conditions are listed below.

- Hydrolysis time: 14–48 h
- Type of enzyme: Maxipro NPU and FPC
- Enzyme Concentration: 20% w/w each
- Enzyme/F1 protein: 40 mg of each
- Concentration of F1 protein: 200 mg in 10 mL H_2O

It should be noted here that Zorbax SB-C_{18} chromatography columns had to be replaced due to poor separation efficiency after long usage. Previous experiments had revealed that XDB-C_{18} had as good as if not better selectivity for the separation of amino acids compared to Zorbax SB-C_{18}. Therefore, all analyses from this point were performed on XDB-C_{18}. Also, it is important to note here that results in Table 8 for pH = 7.0 were obtained with Zorbax SB-C_{18} while the results for pH = 7.8 for 14 and 38 h were obtained with XDB-C_{18} column. Percent hydrolysis for both pH values using optimum conditions were between 55 and 62%. The hydrolysis yield, however, when hydrolysis time increased from 14 to 38 h was increased from 56 to 62%.

Table 6. Effect of reaction time on the amount of hydrolyzed of F1 protein. Reaction conditions: see Table 5.

Reaction time (h)	1	4	12	18	20	24	36	44
Yield amino acid (µg/mL)								
Aspartic acid	30.1	55.3	186.5	184.9	182.8	207.2	241.7	235.9
Glutamic acid	48.0	97.2	212.7	221.1	190.4	226.4	277.4	282.0
Asparagine	40.3	78.2	129.6	131.4	123.1	124.8	123.1	109.9
Serine	10.5	49.9	136.9	143.6	135.8	150.4	165.2	169.2
Histidine	10.5	39.3	95.2	101.6	112.0	97.5	92.3	78.5
Glycine	39.9	61.7	92.1	100.1	90.6	104.4	122.5	136.4
Threonine	28.0	87.9	236.8	243.2	206.1	239.0	282.2	299.6
Arginine	66.2	138.2	236.4	245.4	222.9	237.1	239.9	160.7
Alanine	86.1	134.3	285.2	284.0	281.8	294.2	322.8	314.4
Tyrosine	65.8	171.4	355.4	344.1	323.0	341.7	290.1	405.0
Lysine	134.4	221.3	343.5	364.6	305.9	370.8	392.1	410.0
Valine	145.7	259.9	475.6	477.6	456.9	453.4	490.6	478.5
Tryptophan	70.8	102.3	88.0	83.4	78.7	81.9	87.4	85.8
Isoleucine	80.7	161.7	333.6	328.2	322.0	315.4	339.5	329.1
Leucine	183.4	301.6	456.0	453.2	422.3	445.2	470.9	466.8
Total weight (mg/mL)	1.04	1.96	3.66	3.71	3.45	3.69	3.94	3.96

Table 7. Effect of air on hydrolysis of F1 protein. Reaction conditions: see Table 5.

Reaction environment	Anaerobic	Aerobic
Yield amino acid (µg/mL)		
Aspartic acid	150.0	182.8
Glutamic acid	198.2	190.4
Asparagine	115.5	123.1
Serine	128.5	135.8
Histidine	91.6	112.0
Glycine	97.2	90.6
Threonine	198.2	206.1
Arginine	237.0	222.9
Alanine	255.2	281.8
Tyrosine	327.1	323.0
Lysine	353.2	305.9
Valine	442.2	456.9
Tryptophan	79.7	78.7
Isoleucine	301.7	322.0
Leucine	416.4	422.3
Total weight (mg/mL)	3.39	3.45

Table 8. Results for hydrolysis of F1 protein by varying more than one parameter at the same time. Reaction conditions: see Table 5.

Reaction parameters	ph 7.0 50 °C 48 h	ph 7.8 47 °C 14 h	pH 7.8 47 °C 38 h
Amino acid (µg/0.5 mL)			
Aspartic acid	487.2	228.6	308.9
Glutamic acid	484.4	375.5	569.2
Asparagine	193.3	282.5	244.5
Serine	288.9	226.6	271.9
Histidine	113.8	317.8	289.3
Glycine	203.4	176.4	209.2
Threonine	459.7	0.0	0.0
Arginine	263.9	567.2	597.8
Alanine	426.5	334.5	413.7
Tyrosine	350.5	768.9	788.6
Lysine	511.3	184.0	187.0
Valine	586.2	608.4	690.3
Tryptophan	121.5	605.2	604.7
Isoleucine	425.6	383.2	454.9
Leucine	562.6	532.4	591.1
Total weight (mg/0.5 mL)	5.48	5.59	6.22

Elution order of threonine and glycine changed when SB-C18 was replaced with XDB-$C_{18.}$ Threonine was not detected. It was possibly eluted with Arginine.

Also, the yield of some amino acids after hydrolysis was somewhat altered when the analytical column was switched from SB-C_{18} to XDB-C_{18}. It appeared that improved resolution of the internal standard may have yielded improved quantitation.

F1 protein conversion versus reaction volume and protein amount

Using the optimized enzymatic hydrolysis conditions (20% w/w enzyme/F1 protein, pH = 7.5–7.8, temperature = 45–47 °C, and 14–18 h, 20 mL DI water for every gram of F1 protein), and a sample load of 10 g of F1 protein with appropriate reagent adjustments, conversion of the F1 protein to free amino acids was at least 56% (Table 9). This 56% conversion is a conservative value since proline and glutamine were not determined and these two amino acids are noteworthy constituents of tobacco F1 protein. The qualitative array of free amino acids appearing in Table 9 are also very consistent with the amino acid composition of F1 protein as well as with results of the previous experiments. Tables 10–12, show the quantity of free amino acids when 40 g F1 protein per batch was hydrolyzed (total of 3 batches) employing optimized conditions. Figure 4 is a representative chromatogram of each batch. The average conversion rates of higher than 50% are very similar to published conversion rates of vegetable protein using enzymatic technologies (14).

Table 9. Enzymatic conversion of 10 g F1 protein to free amino acids. Hydrolysis conditions: 20% w/enzyme/F1 protein, pH 7.5–7.8, temperature 45–47 °C, hydrolysis time 14–18 h, 20 mL DI water/g of F1 protein.

Hydrolysis time	14 h
Amino acid (µg/200 mL)	
Aspartic acid	305.4
Glutamic acid	479.0
Asparagine	225.6
Serine	230.6
Histidine	349.3
Threonine	ND
Glycine	165.6
Arginine	548.6
Alanine	347.1
Tyrosine	547.1
Lysine	316.9
Valine	664.7
Tryptophan	296.8
Phenylalanine	285.8
Isoleucine	381.0
Leucine	533.6
Total weight of amino acids (mg/200 mL)	5676.8

Elution order of threonine and glycine changed when SB-C18 was replaced with XDB-$C_{18.}$ Threonine was not detected. It was possibly eluted with Arginine. ND: not detected

Isolation of free amino acids

For the first part of this study, a 50-µL mixture of 15 amino acids (10 nmole/µL per each for a total of about 1 mg) standards was loaded separately into two conditioned columns (with approximately 6–7 g of resin). The amino acids loaded into each column were washed and eluted. After elution, the solvent was evaporated using a rotary evaporator, and the residue was re-dissolved in 2 mL of 0.1N HCl for HPLC analysis. Table 13 shows the recovery of amino acids loaded onto the column, after they were washed, eluted, and analyzed via HPLC. Recovery for most amino acids was above 80%. However, poor recoveries of arginine, tyrosine, and tryptophan were observed. When similar experiments with a larger mass of amino acids (100 µL: 2.5–3.0 mg) were conducted, a lower recovery of amino acids was observed compared to the same column when an amount of 50 µL was loaded into the column. These experiments indicated that 100 µL of standard amino acids exceeded the capacity of this resin to trap the amino acids. Table 13 shows the results of this study in duplicate.

Table 10. Batch 1 AA analysis results of hydrolyzed F1 protein (40 g) in 1 L.

Amino acid	mg/L	Distribution of AA´s (%)
Aspartic acid	1170.2	5.36
Glutamic acid	1877.8	8.60
Asparagine	721.9	3.31
Serine	903.8	4.14
Histidine	1510.8	6.92
Threonine	814.8	3.73
Gycine	1135.5	5.20
Arginine	414.3	1.90
Alanine	1628.0	7.46
Tyrosine	2646.3	12.12
Lysine	1956.8	8.96
Valine	2357.4	10.80
Tryptophan	183.8	0.84
Phenylalanine	555.8	2.55
Isoleucine	1563.7	7.16
Leucine	2388.4	10.94
Total mass of amino acids (mg)		21,839.13
Percent hydrolyzed		54.57

Table 11. Batch 2 AA analysis results of hydrolyzed F1 protein (40 g) in 1 L.

Amino acid	mg/L	Distribution of AA's (%)
Aspartic acid	1269.2	5.70
Glutamic acid	1971.3	8.86
Asparagine	692.1	3.11
Serine	843.7	3.79
Histidine	1788.7	8.04
Threonine	847.9	3.81
Glycine	1085.8	4.88
Arginine	382.3	1.72
Alanine	1618.2	7.27
Tyrosine	3068.2	13.78
Lysine	1869.7	8.40
Valine	2338.0	10.50
Tryptophan	280.7	1.26
Phenylalanine	424.2	1.91
Isoleucine	1531.8	6.88
Leucine	2245.5	10.09
Total mass of amino acid (mg)		22,258.22
Percent hydrolyzed		55.64

Table 12. Batch 3 AA analysis results of hydrolyzed F1 protein (40 g) in 1 L.

Amino acid	mg/L	Distribution of AA's (%)
Aspartic acid	1030.7920	5.18
Glutamic acid	1667.1510	8.38
Asparagine	595.5866	2.99
Serine	797.1154	4.01
Histidine	1151.9600	5.79
Threonine	748.9085	3.76
Glycine	1086.3550	5.46
Arginine	297.7753	1.50
Alanine	1544.1980	7.76
Tyrosine	2409.2440	12.10
Lysine	1769.3990	8.89
Valine	2229.8180	11.20
Tryptophan	272.2601	1.37
Phenylalanine	650.1111	3.27
Isoleucine	1470.7940	7.39
Leucine	2181.6370	10.96
Total mass of amino acid (mg)		19,903.10
Percent hydrolyzed		49.75

It is important to note here that in each experiment fresh resin was used. Dowex MAC-2 was next tested as a packing material for trapping the amino acids. This resin was prepared and treated similar to the Amberlite IR120. Table 13 contains the percent recovery of each amino acid when Dowex MAC-2 was used in duplicate runs. Recovery was much lower than that obtained for Amberlite IR120.

Trapping efficiency of Amberlite IR120 for hydrolyzed F1 protein

1 g of tobacco-derived F1 protein was enzymatically hydrolyzed using 50 mg of Maxipro NPU and 50 mg of Maxipro FPC in 100 mL of DI water, as described above. Next, the pH of the solution was adjusted to 7.0 using 1M NaOH or 1M HCl after which the solution was placed in a heated bath at 50 °C for 24 h. After completion of the reaction, the solution was heated at 85˚C for 10 min to deactivate the enzyme. Under these hydrolysis conditions, HPLC analysis showed that only 31.4% of the protein was hydrolyzed.

In order to show the trapping efficiency of Amberlite IR120, different volumes of the hydrolyzed F1 protein solution (200, 400, 600, 800 and 1000 µL) were loaded onto various columns. Each column was prepared according to our experimental procedure. After loading the sample onto the columns, the samples were washed and eluted according to the previously described procedure. Figure 5 shows the linear trapping efficiency of resin for amino acids from 200–800 µL of hydrolyzed F1 protein solution. Figure 4 shows a representative chromatogram of amino acids obtained via hydrolyzed F1 protein.

A similar experiment was investigated to isolate the free amino acids from 5 mL of hydrolyzed F1 protein. In this study a larger column (200 × 25.4 mm) was packed with 60–65 g of Amberlite IR120. After preparation of the column, a hydrolyzed sample (5 mL of hydrolyzed F1 protein filtered and mixed with 50 mL of 0.1 N HCl) was loaded and the column was treated according to the procedure explained previously except everything in this experiment was multiplied by 5. Table 14 shows percent recovery of each amino acid after 5 mL of hydrolyzed F1 protein was passed through Amberlite IR120. Most amino acids showed recovery higher than 90%, although histidine and lysine recoveries were 66 and 76% respectively. Recovery of threonine was 53%.

CONCLUSIONS

Conditions for the optimized enzymatic hydrolysis of tobacco-derived F1 protein producing free amino acids have been described. The optimized enzymatic hydrolysis

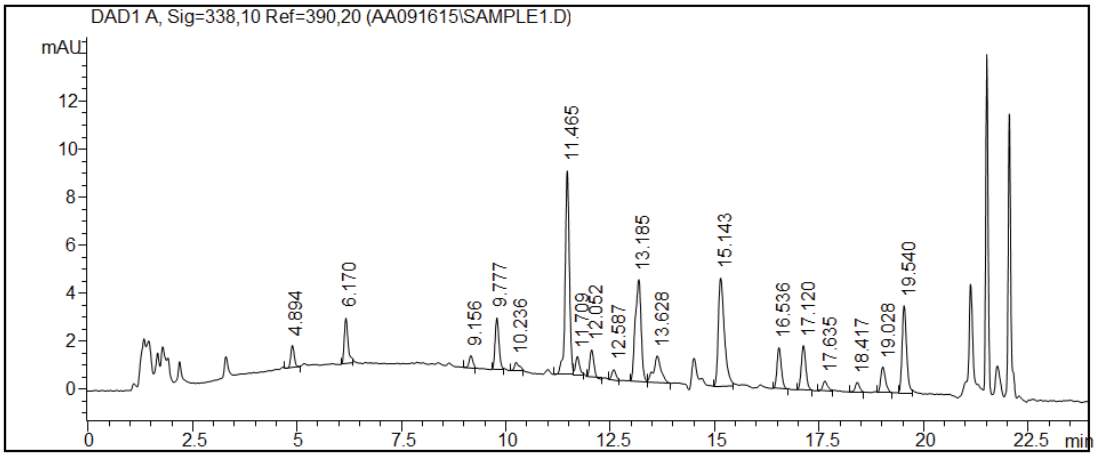

Figure 4. Typical HPLC separation of free amino acids from enzymatic hydrolysis of F1 protein.

Table 13. Duplicate results for percent recovery at two different amounts (µL of standard solution) of amino acids loaded into Amberlite IR120 and Dowex MAC-2.

AA Loading	Amberlite 50 µL Recovery (%)	Amberlite 50 µL Recovery (%)	Amberlite 100 µL Recovery (%)	Amberlite 100 µL Recovery (%)	Dowex 50 µL Recovery (%)	Dowex 50 µL Recovery (%)
Aspartic acid	92.6	96.3	75.8	73.2	13.0	14.1
Glutamic acid	80.0	81.8	63.0	64.5	22.7	21.9
Asparagine	85.8	89.7	75.8	71.9	16.0	21.2
Serine	99.3	104.7	87.6	83.6	13.6	14.1
Histidine	75.7	85.2	57.4	67.2	33.2	12.7
Glycine	112.3	118.7	86.6	85.0	14.1	13.1
Threonine	86.2	89.3	79.1	73.2	10.4	15.2
Arginine	27.6	49.7	15.7	36.1	20.9	28.5
Alanine	102.9	102.9	92.3	85.6	10.3	19.1
Tyrosine	55.7	55.7	46.2	58.4	67.0	43.9
Lysine	95.5	108.1	45.7	65.5	27.6	17.9
Valine	89.5	101.7	73.4	81.3	22.8	20.8
Tryptophan	42.6	58.5	29.1	46.2	58.7	71.4
Isoleucine	89.7	98.6	64.1	71.8	23.5	30.9
Leucine	79.4	86.3	64.0	72.5	21.3	28.8

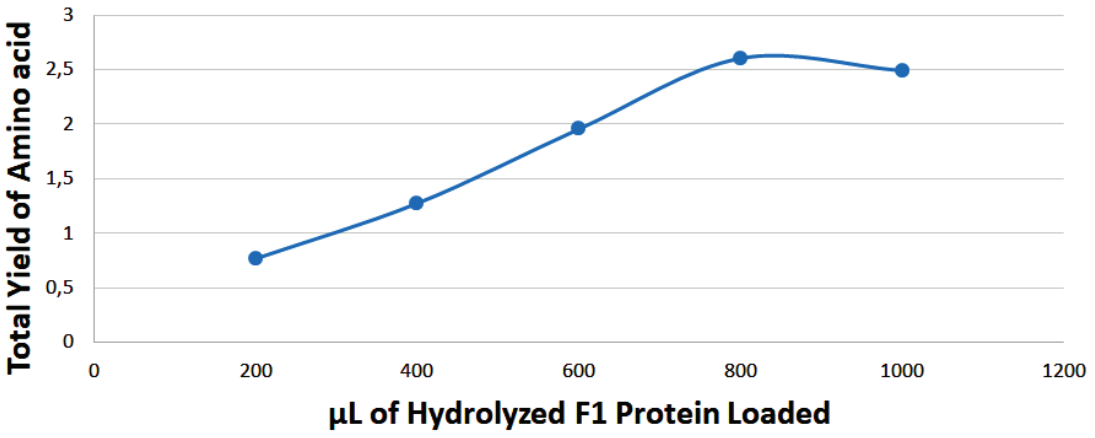

Figure 5. Trapping efficiency of Amberlite IR120 resin for amino acids from hydrolyzed F1 protein. Hydrolysis conditions: 200 mg of F1 protein, 20 mL of H_2O, 50 mg of each Maxipro NPU and FPC, pH = 7.0, heated at 50 °C for 24 h.

Table 14. Percent recovery of different amino acids from 5 mL of hydrolyzed F1 protein loaded into Amberlite IR120.

Amino acid	Recovery (%)
Aspartic acid	100.7
Glutamic acid	100.7
Asparagine	95.2
Serine	107.6
Histidine	66.0
Glycine	104.8
Threonine	53.0
Arginine	93.9
Alanine	111.4
Tyrosine	90.0
Lysine	76.4
Valine	103.1
Tryptophan	111.6
Phenylalanine	65.1
Isoleucine	93.2
Leucine	89.7

conditions included systematic variations in the following: enzyme concentration, reaction temperature, reaction time, reaction pH, enzyme type, reaction atmosphere, protein concentration, as well as a few experiments varying multiple optimized parameters at once. With the exception of reaction environment, that is aerobic versus anaerobic, significant impact on enzyme efficiency was noted for each parameter examined. When fully optimized on these parameters, the conversion of F1 protein to free amino acids was consistently greater than 50%. Amberlite IR120 resin was shown to more effectively trap the free amino acids from the hydrolyzed F1 protein solution when compared to Dowex MAC-2 resin. More specifically, the preconditioned resin was able to efficiently trap ~1 mg of free amino acids from the hydrolysis solution per 4 g of resin when employing conventional gravity fed, glass chromatographic columns. Free amino acid trapping efficiencies were linear up to ~2.5 mg for a 10 mm column with different efficiencies being noted as a function of specific amino acids, in particular arginine, tyrosine, and tryptophan have demonstrated lower trapping efficiencies. Relatively inexpensive analytical methodologies were developed for the rapid quantitative and qualitative analysis of the free amino acids liberated during hydrolysis. Thus, employing optimized reaction parameters, commercially available enzymes have been shown to be effective (50% efficiency) reagents for the hydrolysis of tobacco F1 protein.

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Corresponding author:

Mehdi Ashraf-Khorassani
Department of Chemistry
Virginia Tech
Blacksburg, VA 24061, USA
E-mail: mashraf@vt.edu