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Keywords: Swiss mice, ready-to-use enteral feeding product, in vivo digestibility, pepsin digestibility, hydrolyzed protein

Abstract:

A ready-for-use enteral feeding product was manufactured based on energy standard mixing using hydrolyzed products from local foods (i.e., loin pork, carrot, pumpkin, soybean, rice, and potato). When compared to enteral foods based on defined ingredients, the manufactured product is more cost-effective, appropriately functional and has similar physicochemical properties. Relative protein digestibility for in vitro value was tested by using enzyme pepsin, pH-drop and pH-stat method with three different enzymes. The product was shown to be easily digested with an in vivo digestibility value of 89.7%. Molecule sizes of components in the product ranged from 3.5 to 8.5 kDa, determined by SDS-PAGE, and the average molecular weight was 1.52 kDa, determined by Gel Permeation Chromatography (GPC) method. In terms of nutritional value, the product achieved a caloric density of 1 kcal/mL, dietary fibers of 1.48 g per 100 mL and provided both oligomeric and monomeric forms of protein. In addition, the product has the leucine: isoleucine: valine ratio of 2:1:1, thus facilitating the absorption of the protein. In conclusion, the manufactured enteral feeding product has been shown to be appropriate for providing nutritional support for patients.

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Keywords: hydrolyzed protein; pepsin digestibility; in vivo digestibility; ready-to-use enteral feeding product; Swiss mice

1. Introduction

In parallel with the rapid development of nutritional supplements, products for enteral nutrition support have become increasingly common and are widely applied in routine clinical practice, thus contributing to the reduction of mortality in the treatment of severe patients [1–4]. Enteral foods are a class of liquid foods fed to patients though a catheter under medical indication and control. Enteral nutrition (EN) is a safe, effective and generally well-tolerated means of administering nutritional therapy to the normally functioning gastrointestinal tract (GI) of patients who are unable to eat or swallow. Recently, the increased diversity and popularity of commercial EN formulas has contributed

to the improvement in the nutritional status of patients and reduced hospital stays [5–9]. However, in order to be used effectively, EN formulas should meet strict requirements. According to the European Society for Clinical Nutrition and Metabolism (ESPEN) guideline, a standard formula should not pose the risk of tube clogging and infection and should satisfy basic criteria of safety and efficacy [10]. At present, the ready-for-use enteral feeding products with liquid formula, which are now widely available on the market, are often delivered to patients in hospitals and at home. In addition, the pressing demand for better nutritional support during and after sickness period has therefore urged for development, processing and manufacturing of enteral products with reduced intolerances, efficacy and, most importantly, affordability.

Enteral formula is preferred to hospital-made blenderized feed in developing countries. In Vietnam, the majority of EN feeding products used are often made by their caregivers, and therefore, may not meet standard requirements of quality and safety. In addition, the dilution of the pureed homemade product required for passage though the catheter might lead to lowered energy density and the loss of nutrients and pose high risk of microbial contamination. However, despite these limitations of self-made EN, the availability of high-quality EN products in central hospitals of Vietnam is still limited, mostly due to the high cost of imported EN and the lack of a reliable source of domestically manufactured products.

A feeding product should first meet the nutritional criteria, especially protein in general and animal protein in particular. The latter, along with carbohydrates, serves as the main source of energy. In addition, the product should also be hygienic, tailorable to the variety of forms such as liquid (use for tube feeding) and solid (for swallowing), and affordable. Other important quality indicators could include digestibility, which depends on isolated protein and its density, and absorption, measured by comparing nitrogen in food intake and in stool. For protein digestibility, many in vitro assays have been developed for quick estimation of protein digestibility and replacement of feeding trials in animals including the pH drop, pH stat and pepsin digestibility with single/multi enzyme with one/two steps digestion. Even though the use of these methods has been associated with improved in vivo protein digestibility [11–16], biological experiments for in vivo assays are complex and expensive. This urges for the use of more convenient approaches for assessment of protein digestibility. In this study, three in vitro methods including the pepsin digestibility, the pH stat and pH drop with three enzymes, were chosen to determine the protein digestibility of the manufactured product. While the justification for pepsin digestibility is made by the popularity of the technique and the official approval of Association of Official Analytical Chemists (AOAC method of 971.09), the pH stat and pH drop are selected mainly due to the ease to performance, good reproducibility and agreement with results produced by in vivo methods.

These issues led to the development of a new enteral diet which is both nutritionally adequate for post-operative patients (i.e., patients with high calorie and protein demands) and affordable for patients in developing countries. The successful implementation of such products could address the shortage of enteral food for tube feeding in hospitals and relieve the financial burden expended on costly feeding formula for families of such patients. In this study, a liquid feeding product from locally available ingredients in Vietnam was developed and tested for absorption and digestibility via either in vitro or in vivo assay.

2. Materials and Methods

2.1. Preparation of Enteral Feeding Product

The feeding product was prepared from local ingredients. To be specific, 190 g of loin pork meat was pureed in 1.5:1 ratio to water, heated to 100 °C, and maintained at this temperature for 10 min under stirring. Following that, the mixture was allowed to cool naturally. When the temperature reached 64.6 °C, the pH of the mixture was adjusted by 7.5 with 10% Na_2CO_3 solution. At 64.5 °C, Alcalase with a concentration of 1.77% (*v*/*w*) and enzyme activity of 31.53 U/g was added to the mixture,

followed by hydrolysis for 256 min. This duration is necessary to achieve the minimum viscosity of the hydrolyzed product of 2.41 cP.

Other ingredients including 500 g of carrots, 500 g of pumpkin, and 100 g of potatoes were soaked, washed, peeled and cut into 1 × 1 × 1 cm cubes. Carrots and pumpkins were cooked at 100 °C for 6 and 4 min, respectively, followed by hydrolysis by Pectinex at a concentration of 3.9% (*v*/*w*) with enzyme activity of 46 U/g at 43 °C in 127 min and at pH of 4.6. These conditions are necessary to achieve the minimum viscosity of the product at 9.4 cP. For rice and potatoes, 100 g of each ingredient was mixed and cooked at 100 °C for 15 min. The mixture then was hydrolyzed by Termamyl SC enzyme at the concentration of 2.9% (*v*/*w*) at 89 °C for 160 min, pH of 6.6 and under stirring. Specifically for soybeans, 50 g of soybeans was soaked with warm water (40 °C) for 4 h. Soybeans were then cooked at 100 °C for 30 min and were hydrolyzed by Alcalase enzyme with a concentration of 1.5% at 55 °C in 180 min and at a pH of 7.

All the ingredients after hydrolysis were mixed and added with a mixture consisting of 10 g of soybean oil, 10 g of medium chain triglycerides (MCT) and 3.5 g of salt. Following that, the temperature was elevated to 70 °C and the mixture was pumped through the 0.4 mm filter. In the filter pot, the hydrolysate was stirred for 5 min and then homogenized into two levels at 150 bar and 50 bar. UHT pasteurization was performed at 140 °C for 15 s and the obtained products were packed into four plastic bottles (400 mL capacity) for further analysis.

2.2. Proximate Analysis

The protein of the enteral feeding product was quantified by the Kjeldahl (FAO FNP 14/7) with a conversion factor of 6.25. For the extraction of fat, Soxhlet extraction was performed with petroleum ether. The carbohydrate content was determined by AOAC 986.25 mod. The contents of the pesticide and microorganism were determined by the AOAC method of 969.08 and 994.03, respectively [17,18]. The contents of K, Fe, Mg, Na, and Ca were determined by the AOAC method of 2011.14 [19]. Total phosphorus and Zn content were determined by the AOAC method of 995.11 and 999.11, respectively [20,21].

2.3. Analysis of the Amino Acids in Samples of Protein Concentrate (100 mg)

For the determination of the amino acids, the samples were determined by AOAC 994.12 (GC-FID) [22]. The prepared samples were run on a Clarus 680 Gas Chromatograph (Perkin Elmer, Waltham, MA, USA) with a flame ionization detector. Precolumn: 60 m × 0.530 mm × 5 µm PerkinElmer Elite 1 with 25 cm × 0.250 mm deactivated fused silica restrictor connected between S-Swafer and column. Analytical Column: 10 m × 0.530 mm × 10 µm Varian Lowox with in-line 25 cm × 0.100 µm deactivated fused silica restrictor connected between S-Swafer and column. Lab conditions at 22 °C, detector temperature was 250 °C. The oven temperature was programmed from 100 °C to 275 °C (maintained for 8 min) with a ramp of 40 °C/min. Carrier gas was He, with an inlet pressure of 115 kPa and 1:20 split ratio. The volume of the sample injected was 5 µL. Amino acids composition was expressed as g/100g of protein.

2.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli method was employed to conduct SDS-PAGE with 12% separating and 4% stacking gels using low molecular weight (3.5–38 kDa) markers obtained from GE Healthcare Rainbow™ Molecular Weight Markers (catalog number 45-001-593, Fisher Scientific, Hampton, NH, USA).

2.5. Gel Permeation Chromatography Method

Average molecular weight was determined based on the retention time of each type of chromatography column. The retention time of the polysaccharide has an average molecular weight difference obtained from the different gel chromatography. After comparing with the retention time of the standard sample, the molecule weight and the corresponding segment could be determined. In the

GPC method, three indicators could be calculated including the number average molecular weight (M_n), weight average molecular weight (M_w) and maximum dispersion index (D).

M_n: Number average molecular weight;
M_w: Weight average molecular weight;
M_z: Z-average molecular weight;
M_v: Viscosity average molecular weight.

The number average molecular weight, M_n, in the sample is calculated as follows.

$$M_n = \frac{W}{\sum N_i} = \frac{\sum N_i M_i}{\sum N_i} \quad (1)$$

where:

Size molecules with different distribution from $i = 1 \rightarrow \infty$;
N_i the number of moles (or molecules) molecular weight M_i;
$W = \sum M_i N_i$: total mass of the polymer;
$\sum N_i$: total moles of polymer.
M_w: Weight average molecular weight. M_w is calculated as follow:

$$M_w = \sum P_i M_i \quad (2)$$

where P_i is a mass of molecules in molecular weight M_i

$$P_i = \frac{W_i}{\sum W_i} = \frac{W_i}{W} \quad (3)$$

$$W = \sum W_i = \sum N_i M_i \quad (4)$$

$W_i = N_i.M_i$.

2.6. Protein Digestibility by Pepsin

In Vitro digestibility assessment was performed according to the method previously described by Siccardi III and AOAC 971.09 [23,24]. To be specific, initial samples were de-fatted by Soxhlet extraction with petroleum ether. Following that, 0.5 g of defatted sample was subject to rotating machines on the tube together with 150 mL of 0.0002% pepsin solution (in 0.075N HCl) (pepsin purity, activity 1:10,000, RM1251, Sigma-Aldrich, St. Louis, MO, USA). The mixture was then incubated at 45 °C for 16 h under rotation of 15 rpm. After incubation, for the clarification, the mixture was filtered with a filter paper through a Buchner funnel, then it was rinsed twice with acetone (15 mL of acetone). The determination of obtained crude protein residue (crude protein of undigested residue) is as follows.

$$\text{Protein digestibility } (\%) = 100 - \frac{A}{B} \times 100 \quad (5)$$

where:

A: total crude protein content in residue obtained (mg);
B: total crude protein content in sample (mg).

2.7. pH-Drop and pH-Stat Method for Prediction of Protein Digestibility in Foods

During the proteolysis, a reduction in pH may occur due to the release of protons from the cleaved peptide bonds. Since the initial rate of peptide release and protein digestibility are associated,

the protein digestibility could be calculated from data of pH decline (pH-drop method) or NaOH consumption (pH-stat method) given the constant pH and continual addition of NaOH.

The enzyme preparation used in the in vitro study was either an isolated enzyme or an enzyme mixture. In both cases, enzymes were purchased from suppliers as follows. Trypsin from pig pancreas, type IX, activity 13,000–20,000 BAEE units/mg protein (catalog number T0303) and α-chymotrypsin from bovine pancreas, Type II, activity ≥ 40 U/mg protein (catalog number C4129) were from Sigma-Aldrich; Flavourzyme ®500 mg, activity 500 LAPU/g was from Novozymes (Bagsværd, Denmark).

The 3-enzyme pH-drop method was performed following previous studies [25,26]. First, 18.75 mg of protein samples was introduced into 10 mL of distilled water in a cup. The mixture was well shaken for 1 h at room temperature. Then the pH was adjusted to 8.0 with NaOH 0.1 N. Following that, the enzyme mixture containing 1.6 mg·mL^{-1} of trypsin, 3.1 mg·mL^{-1} chymotrypsin and 1.6 mg·mL^{-1} of Flavourzyme® 500 mg was added. A pH meter (Sensions3, HACH) was used to record the pH at 1-minute intervals for 10 min. The protein digestibility was calculated with casein as the reference protein. To be specific, the relative protein digestibility (RPD) of enteral food was calculated by the following equation.

$$\text{RPD } (\%) = \frac{-\Delta\text{pH of ingredient}}{-\Delta\text{pH casein}} \times 100 \quad (6)$$

where −ΔpH of the ingredient was the percentage change of pH drop, and −ΔpH casein was the pH drop of the casein enzyme [25].

In the pH-stat method, the pH was kept constant by automatic titration with NaOH 0.1 N. After the incubation, a specific amount of alkali was added and recorded [27]. In the pH stat, the reaction was initiated by the enzyme system mixture (as above) (pH adjusted to 8 using NaOH 0.1 N) and allowed to continue for 60 min. Following that, the mixture containing the enzyme and protein was titrated using NaOH 0.1 N from the automatic voltage titration Tritra-Lab Radiometer 865. The required amount of NaOH for the reaction mixture at pH 8 for 60 min was recorded. Degree of hydrolysis (DH) was calculated following Adler-Nissen (1986) and Cordova-Muruete and Garcia-Carreno (2002) [28,29] as follows:

$$\text{DH } (\%) = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100 \quad (7)$$

where:

B: the amount of standard Alkali (NaOH 0.1 N) consumed to maintain the reaction mixture at pH 8 (mL);
N_B: the normality of the titrant (concentration of NaOH equivalents) (N);
α: average degree of dissociation of the α-NH groups.
MP: mass of substrate protein (%);
h_{tot}: total number of peptide bonds in the protein substrate according to the source of protein (meq/g).

2.8. In Vivo Digestibility on Swiss Mice

For the assessment of protein digestibility and quality of the enteral product, the mice feeding experiment was performed. Subjects of the study were 20 healthy male Swiss albino mice with a weight of 21.48 ± 1.19 g provided by HCMC Institute of Drug Testing and Control, Ho Chi Minh City, Vietnam. The experiments were conducted on batches of 10 mice housed together. The mice were acclimatized and reared on manufactured food for 05 days with one feeding a day at 1:00 p.m. before being put on the equinox. On the sixth day, the experimental feeding commenced on all mice and lasted for 7 days. The feeding formula in the experiment consisting of 1 mg of Cr_2O_3 (99% purity, Sigma-Aldrich) was blenderized in 100 mL of the manufactured enteral feeding product. The excreta were collected daily and stored at −20 °C during the experiment. At the end of the experiment, the body weights of mice were recorded and excreta samples were weighed and analyzed for total nitrogen. The digestibility was determined using protein intake and fecal data.

Digestibility assessment using an in vivo method involves comparing data on animal feeding fecal data. Mouse feces obtained after drying at 50 °C for 24 h were then determined for the moisture, crude protein content and Cr_2O_3. Cr_2O_3 is the inert marker. The Cr_2O_3 content is determined by the method of Furukawa & Tsukahara [30]. Apparent protein digestibility (APD) was calculated using the following equation [31]:

$$\text{APD } (\%) = \frac{\%\ Cr_2O_3 \text{ in food}}{\%\ Cr_2O_3 \text{ in feces}} \times \frac{\%\ \text{protein in feces}}{\%\ \text{protein in food}} \times 100 \quad (8)$$

where:

% Cr_2O_3 in food: % Cr_2O_3 calculated on dry matter feed;
% Cr_2O_3 in feces: % Cr_2O_3 calculated on dry matter in feces;
% Protein in food: % crude protein on dry matter of feed;
% Protein in feces: % crude protein calculated on dry matter in feces.

2.9. Statistical Analysis

The data were processed using Microsoft Excel software. All experiments were repeated at least three of replica. The experimental results presented are the average values of the repetitions. Data were analyzed by ANOVA, and differences were considered significant when $p < 0.05$ calculated by Statgraphics Centurion XVI.

3. Results

3.1. Energy Content and Nutrition Contents

The nutrient composition of the enteral food is presented in Table 1. The bulk density of the enteral food was 1.08 g·mL^{-1} and the pH was 6.88. The enteral food provided 1 kcal per ml and had desirable taste and odor. The product was also smooth in texture and creamy yellow in color. Regarding nutrients, the protein, fat and carbohydrate content were 4.03 ± 0.03%, 2.94 ± 0.02% and 11.9 ± 0.08% respectively.

Table 1. Nutritional composition of the manufactured enteral feeding food.

Composition	Content	Vitamin	Content
Protein	4.03 ± 0.03%	Vitamin A	46.2 µg/100 mL
Fat	2.94 ± 0.02%	Vitamin E	2.91 mg/100 mL
Carbohydrates	11.9 ± 0.08%	Vitamin D3	1.87 µg/100 mL
Soluble fiber	0.37%	Vitamin B1	0.17 mg/100 mL
Insoluble fiber	1.11%	Vitamin B2	0.22 mg/100 mL
Na	120.8 mg/100 mL	Vitamin B3	1.54 mg/100 mL
K	193.5 mg/100 mL	Vitamin B5	0.60 mg/100 mL
Ca	64.5 mg/100 mL	Vitamin B6	0.19 mg/100 mL
P	56.1 mg/100 mL	Vitamin B9 (acid folic)	21.2 µg/100 mL
Mg	20.9 mg/100 mL	Biotin	16.5 µg/100 mL
Fe	1.68 mg/100 mL	Vitamin B12	0.51 µg/100 mL
Zn	2.67 mg/100 mL	Vitamin C	19.1 mg/100 mL
Iodine	14.7 µg/100 mL		
Viscosity	83 cP		

3.2. Amino acid Analysis

Table 2 showed the amino acids content of the enteral food including specific contents of other amino acids, aromatic amino acids, branched chain amino acids (BCAA), and sulfur amino acids. The amino acid content of the products used in feeding tube is determined by high-performance liquid chromatography. Protein composition contains 18 kinds of acid amino acids. Essential amino acids (Val, Leu, Ile, Thr, Phe, Lys, Tryp, His and Met) collectively occupied the highest percentage of 43.9%.

Branched chain amino acids predominantly came from pork loin and soybean [32,33]. Branched chain amino acids of the feeding product had leucine of 0.29/100 g, isoleucine of 0.17/100 g, and valine of 0.17/100 g equivalent to the leucine: isoleucine: valine ratio at 2:1:1.

Table 2. Amino acid content of the enteral food.

Amino Acids		Content (g/100 mL)
Other amino acids	Acid Aspartic	338.5
	Serine	153.2
	Glycine	213.2
	Histidine	166.7
	Acid glutamic	607.6
	Threonine	168.7
	Alanine	251.5
	Proline	202.9
	Lysine	306.4
	Tryptophan	56.2
	Acid glutamic	607.6
Aromatic amino acids (AAA)	Tyrosine	176
	Phenylalanine	197.7
	Total AAA	373.7
Branched chain amino acids (BCAA)	Leucine	259.8
	Isoleucine	171.8
	Valine	169.8
Sulfur amino acids	Cysteine	13.04
	Methionine	0.11
	Arginine	950.2

3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoretic patterns were determined by SDS-PAGE as shown in Figure 1. It was indicated that the enteral food mostly constituted of peptides whose molecular weights were lower than 8.5 kDa. In addition, a broad range of medium-size and low molecular weight polypeptides were also determined. Therefore, the product could be easily digested and is suitable for subjects who are in post-operation or in a period of gastro-intestinal tract recovery.

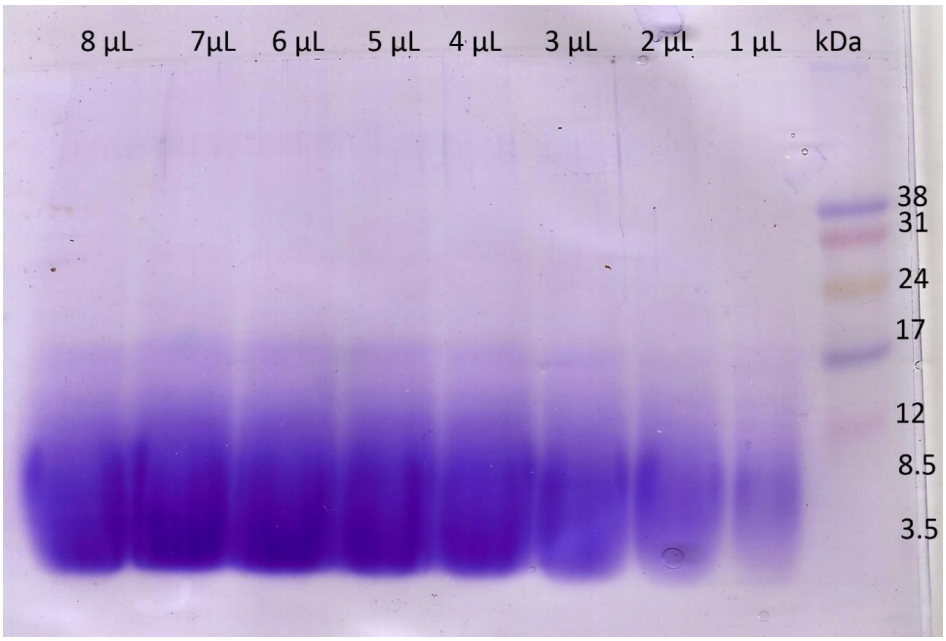

Figure 1. SDS-PAGE pattern of enteral food.

3.4. The Molecular Weight of the Enteral Feeding Product by Gel Permeation Chromatography Method (GPC)

The molecular weight averages of the ready-to-use enteral product were analyzed by gel permeation chromatography method. The molecular weight of the sample was distributed as in the mass distribution window in Figure 2. The retention time of the sample is 11.856 min. This corresponds to M_w, M_n and M_w/M_n ratio of 1.52 kDa, 0.623 kDa and 2.43, respectively.

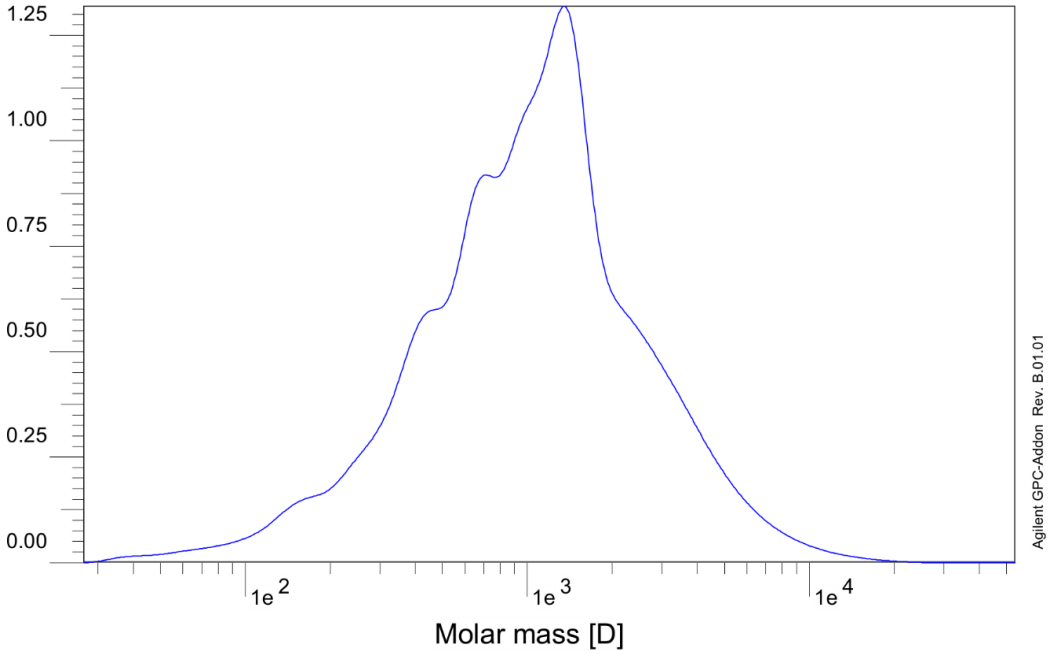

Figure 2. The gel permeation chromatography graph of enteral food.

3.5. Evaluation of Digestibility

Results of in vitro pepsin digestibility are shown in Table 3. The digestibility value for pepsin of the product was 80%, which is relatively high.

Table 3. Digestible protein content and digestibility value for pepsin of the enteral feeding product.

Dry Matter Content (%)	Content
Digestible protein (g/100 g)	4.0
Digestibility value for pepsin method (%)	80.0

The pH-stat results of the enteral feeding product are shown in Table 4. The average amount of NaOH used is 1 mL. The protein absorption calculated according to Equation (7) is shown in Table 5.

Table 4. pH-stat results of the enteral feeding product.

Times	pH of Sample	pH (after Enzyme Added)	NaOH Used after 60 min (mL)
1	6.82	7.55	1
2	6.87	7.57	1
3	6.88	7.58	1

Table 5. Protein absorption of the enteral feeding product.

NaOH Used (mL)	h_{tot}	$\frac{1}{\alpha}$	MP	The Protein Absorption (DH%)
1	8.15	1.4	0.01875	91.62

Table 6 presents the experiment results for the pH-drop method. Initially, the pH of both casein and the enteral product solution was approximately 8.0. The hydrolysis of the feeding product and casein solutions was performed by mixing the enzymes: trypsin, chymotrypsin and Flavourzyme® 500 mg for 10 min at room temperature. After hydrolysis, the pH of casein solutions and the enteral product solution was 7.09 and 7.24 respectively (Table 6). The in vitro RPD of the enteral feeding product was 83.5%, which shows a high rate of protein digestion by using the method of pH-drop with three enzymes.

Table 6. Change of pH in casein and enteral feeding product in relation to hydrolysis time.

Time (Min)	pH Change	
	Casein	Enteral Product
0	8.00 ± 0.000	8.00 ± 0.000
1	7.49 ± 0.005	7.53 ± 0.010
2	7.41 ± 0.005	7.48 ± 0.000
3	7.36 ± 0.000	7.44 ± 0.005
4	7.31 ± 0.005	7.41 ± 0.005
5	7.27 ± 0.005	7.37 ± 0.005
6	7.23 ± 0.010	7.34 ± 0.005
7	7.17 ± 0.005	7.30 ± 0.000
8	7.13 ± 0.005	7.27 ± 0.005
9	7.10 ± 0.010	7.25 ± 0.005
10	7.09 ± 0.005	7.24 ± 0.005

Table 7 presents results of in vivo digestibility assessment, showing the protein and Cr_2O_3 content in the feeding formula fed to mice and in mouse feces. After 1 week of feeding with the enteral product, mice achieved the weight gain of approximately 29.28%. The initial and post-fed weight of mice was 21.48 ± 1.19 g and 27.77 ± 1.29 g, respectively. APD of the enteral product when tested on Swiss albino mice was 89.70%.

Table 7. Protein and Cr_2O_3 content in food and in Swiss mouse droppings.

Dry Matter Content (%)	Food	Feces
Protein	90.145	8.539
Cr_2O_3	0.220	0.250

4. Discussion

4.1. Product Characteristics

Vietnamese patients may find the developed liquid product for tube feeding familiar because the product is based on locally available food and ingredients, which may facilitate its acceptance. Currently, in most hospitals in Vietnam, the enteral feedings are either blenderized diets prepared by caretakers or formulas unreliably prepared by hospitals from various types of foods such as milk, meat, rice, and vegetables. As a result, requirements of essential nutrients, energy and protein might not be fulfilled. This could influence mortality, morbidity as well as the length of hospital stay of patients. On the other hand, homemade food also faces the risk of poor hygiene, microbial contamination and inadequate nutritional value. Our manufactured feeding product satisfies RDA requirements in terms of nutrients and contemplates the conditionally essential nutrients. Further calculation from Table 1 revealed that the caloric density of the product reached approximately 1 kcal/mL, 52% of which is provided by carbohydrates. The product also provided 1.48 g of dietary fibers per 100 mL.

Another important characteristic of enteral formulas is viscosity. The manufactured product achieved the relatively low viscosity of 83 cP, allowing easy adjustment of flow rate to the patient's condition and preventing tube clogging [34]. The viscosity of a product is determined

by various factors such as nutritional composition, used stabilizers, proteins, and polysaccharides [35]. In addition, manufacturing parameters that alter interactions among nutrients such as dispersion, pH, homogenization conditions, and temperature are also important contributors [34]. Therefore, products with similar caloric densities and nutritional compositions may have different viscosities. For comparison, milk has a viscosity of approximately 13 cP; viscosity of soup may vary from 90 to 352; other standardized enteral formulas have viscosities ranging from 53 to 85 [36].

The product is also rich in branched chain amino acids (BCAAs), which are considered 'immune-enhancers' and an important contributor to nutritional values of enteral feeding. To be specific, amino acids found in our product were leucine of 0.26/100 g, isoleucine of 0.17/100 g, and valine of 0.17/100 g, corresponding to the leucine: isoleucine: valine ratio of approximately 2:1:1. Those compounds mainly came from loin pork and soybeans and have been reported to exhibit anti-liver cancer property and are suitable for applications in nutritional supplementation. In addition, The BCAAs could irreversibly degrade by donating their amino-nitrogen in the Krebs cycle, yielding glutamine and alanine which are crucial for the activation of immune cells in liver and spleen [37]. Other discovered functions of BCAA included enhancing ammonia detoxification to glutamine [38], improving mental state in patients with hepatic encephalopathy, preventing cardiac muscle atrophy induced by physical inactivity, stimulating protein synthesis, and reducing protein breakdown and nitrogen loss, both in skeletal muscle and in the myocardium [39].

Regarding the BCAA ratio, the manufactured product had the ratio of BCAA of approximately 2:1:1. This BCAA ratio was demonstrated to be beneficial, as implied by a previous in vivo experiment [35] where pigs were exposed to leucine, isoleucine, and valine at different ratios in 17% crude protein (CP) diets. The results pointed out that a suitable BCAA ratio (in vivo = 1:0.75:0.75; in vitro = 1:0.25:0.25) could offer several benefits including improvements in intestinal morphology, cell proliferation, intestinal AA absorption, and intestinal protein turnover [40]. Our results are also in line with another study where four different BCAA ratios (leucine:isoleucine:valine), including 0.5:1:1, 1:1:1, 1.5:1:1; 2:1:1 and 4:1:1, were tested. The results revealed that the optimal BCAA ratio should vary between 1:1:1 and 2:1:1 [41]. A higher proportion of BCAAs was demonstrated to be suitable for hepatic failure and hepatic encephalopathy patients. In addition, enhanced nitrogen accretion and liver function were found to be positively correlated with BCAA intake in an extended period of time [42]. In a recent study, BCAA catabolism may be shunted to skeletal muscle, where it indirectly leads to FA accumulation and insulin resistance [43].

Molecules in the produced enteral food were below 8.5 kDa, averaging at 1.52 kDa, suggesting the product's suitability for patients with food intolerance to polymeric feeds or with severe impairment of intestinal absorption. This is consistent with a previous study which also reported diarrhea-reducing effects of diets containing peptides in patients with traumatic brain injury [44].

Bioactive proteins are peptides with relatively small molecular sizes and are classified as special protein fractions exerting positive impacts on the conditions on the human body [45,46]. Short peptides entering the human body can be easily metabolized, taking various physiological roles including the ability to control hypertension, antioxidant function, anti-clotting ability, ability connectivity mineral bacteria, and reducing the risk of cardiovascular diseases [47]. The health benefits of bioactive peptides have been reaffirmed by several studies. Specifically, bioactive peptides from soybean protein were shown to aid in the treatment of cancer [48]. In the manufacturing process of the feeding product, the purpose of hydrolyzing soybean and pork with enzymes is two-fold. First, the hydrolysis could facilitate digestibility and enhance nutrient absorption through fragmentation of proteins into products consisting of short, low molecular weight, and soluble peptides. Second, the fragmentation also in turn releases free peptides, allowing them to demonstrate their inherent activities [49]. Taken together, the nutrients in our product were shown to possess the average molecular weight similar to that of the short peptide, thus inducing easier digestion and making the product suitable for patients to recover gastrointestinal function.

4.2. Tests of Digestibility In Vitro and In Vivo

There are two common methods to measure the digestibility in vitro including the pH-drop method and the pH-stat. The advantages of the pH-stat method over the pH-drop method include the insensitivity of the test material against varying buffer capacity, improved prediction accuracy for protein digestibility, and better applicability to different materials. In addition, the reproducibility of the pH-stat method was also demonstrated to be consistent in different studies. For in vivo assessment of digestibility, it is methodologically conventional to analyze the food intake and fecal data of the experimental animals. However, this method is disadvantageous in terms of cost, time and labor.

Overall, the in vitro results showed higher digestibility on the pure protein samples in comparison with results derived from the combined protein sample. This is due to the presence of plant protein in the product. Therefore, improved in vitro digestibility could be observed in samples that are fully made up of animal protein compared to samples mixed by vegetable or plant protein as well as animal protein. On the other hand, results of digestion in vivo showed that the protein powder exhibits very good digestibility in animal subjects, at approximately 89.70%. This is because protein was cleaved to form the circuit with a low molecular weight peptide (about 8.5 kDa). As a result, animal body could digest and absorb more easily in comparison with the use of non-hydrolyzed materials. For comparison, previous studies involving soy protein, fish meal, and crab powder all demonstrated the high protein digestibility of 90.9%, 86.6%, and 66.4% respectively [26,50,51]. Furthermore, digestibility values of various samples from a previous study by Akiyama et al. [52] indicated that casein (99.1%) exhibited the highest digestibility, followed by wheat gluten (98%), gelatin (97.3%), rice (76.4%), and shrimp paste (74.6%) [5]. Therefore, protein digestibility of the produced enteral food is comparable to that of soy protein and fishmeal, and is only surpassed by those of casein, wheat gluten and gelatin.

By comparing the results of protein uptake of the two methods, it is evident that the in vitro method yielded a higher result than the in vivo method because the proteolytic enzyme activity is largely unaffected by in vitro proteolysis and is responsive to environmental factors such as temperature, pH, intestinal microorganisms, and other enzymes in the digestive system. The in vitro RPD of the enteral feeding product was 83.5%, indicating a high rate of protein digestion when tested with the pH drop method with three enzymes. This result is in line with a previous study which revealed that RPDs of meals made from fish, meat and soybean were 78.08, 72.82 and 76.08% respectively [53]. The present result also showed that protein digestibility evaluated by pH-drop method was lower than that in in vivo Swiss mice experiments, contrasting with the expectation prior to the study on mice. This can be explained by the inverse correlation of the pH drop with regards to the ash content in the sample. As a result, it is suggested that the higher mineral content is associated with greater buffer capacity of the sample.

The ability to digest food is also dependent on the characteristics of the experimental animal [13]. In vitro and in vivo tests revealed that the protein digestibility of our enteral food was relatively high. In addition, experiments on animals revealed that the enteral food protein were safe and of good quality. Our study suggested that rice and legumes (carrots, potatoes, pumpkin) along with pork meat, soy and vegetable oils can be utilized to manufacture nutritionally adequate and tailorable enteral feeding food. This has a special implication for the developing and under-developed countries where enormous benefits may be realized with an inexpensive technology for enteral feeding food prepared from locally available ingredients.

5. Conclusions

By utilizing commercial enzymes, an inexpensive enteral feeding formula has been developed based on fresh and locally available ingredients. Appropriate digestibility assays and composition determinations showed that the manufactured product is highly digestible and nutritionally adequate for critically ill patients. Our manufactured product offers three main advantages. First, since the enzymatic hydrolysis of proteins, carbohydrates and pectin results in a product with low viscosity, the passage of the formula through small feeding tubes could be facilitated. Second, the hydrolysis also

releases short and medium peptides with bioactivity, which could enhance product digestibility and exert numerous health benefits for patients. Third, the use of ingredients that are readily accessible, rather than refined components, may reduce intolerances of patients, significantly cut manufacturing costs, and ease the preparation process. This study acts as a precursor for further development of enteral formulas tailored for patients with particular conditions such as kidney failure and diabetes. In addition, further studies may elucidate the relationship between in vitro and in vivo digestibility assessment.

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