International Journal for Multidisciplinary Research (IJFMR)

E-ISSN: 2582–2160, Volume: 4, Issue: 4, July-August 2022

Evaluation of Proprietary MDZenPro Formulation by Zenherb Labs in Mediating Protein Digestion under INFOGEST In-vitro *S*imulated Gastrointestinal Conditions

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Highlights of the Work

(1) The study has evaluated and reported the efficiency of a proprietary formulation named 'MDZenPro' in digesting proteins using an *in-vitro* simulated digestion. model.
(2) MDZenPro was found to enhance the degree of hydrolysis of the proteins. This was supported by the results of SDS-PAGE.
(3) The results of the study confirm the application of MDZenPro in aiding digestion.

Abstract

Protein breakdown by endogenous enzymes in the gastrointestinal (GI) tract results in generation of peptides and amino acids that act as building blocks in essential biological functions. Exogenous proteases possess immense potential as digestive enzyme supplements that can assist protein digestion in the GI system. Plant proteases, in addition to their promising activity, are considered to be safe in nature. The present study evaluated the potential application of a plant protease based proprietary formulation - MDZenPro in digesting raw whey protein, whey protein isolate and plant protein under INFOGEST simulated GI conditions. The gastric and GI digested protein products were analyzed for determining the degree of hydrolysis. The protein profiles were evaluated using SDS-PAGE. The results of the degree of hydrolysis study revealed that MDZenPro facilitated gastric and GI digestion of proteins. This increase in degree of hydrolysis was noted to be higher than that observed in proteins that were not treated with MDZenPro. The SDS-PAGE profile further supported these findings wherein, the MDZenPro treated protein samples displayed low molecular weight fragmented peptides in contrast to the profile of undigested proteins. The present study thus highlights the promising application of 'MDZenPro' as an effective supplement for protein digestion.

Keywords: Protease, Digestion, MDZenPro, Simulated Gastrointestinal Digestion

Introduction

In recent times, digestive enzyme supplements involved in augmentation of gastrointestinal (GI) digestion have gained immense popularity. Such digestive supplements mainly consist of exogenous

enzymes namely proteases, amylases and lipases that aid in the digestion of biomolecules such as proteins, carbohydrates and lipids respectively (Ianiro et al. 2016; Meghwanshi et al. 2020). The naturally occurring digestive enzymes *viz.* gastric pepsin, trypsin and pancreatic lipase degrade proteins, lipids and carbohydrates and facilitate the absorption of nutrients.

Protein, in particular, is considered to be an essential biomolecule as it increases thermogenesis and satiety, provides nutrition, maintains muscle mass, positive net protein balance, lean body mass and healthy immune system (Arentson-Lantz et al. 2015; Kårlund et al. 2019). Proteases catalyze the breakdown of consumed proteins into amino acids and small peptides in the GI tract and, thereby increase their bioavailability (Wang et al. 2020). Denaturation of proteins by acid and their hydrolysis by gastric pepsin and pancreatic proteases are necessary to facilitate their bioavailability in the human body (Amigo et al. 2020). These digestive proteases are extremely important as the products obtained after protein digestion are involved in vital processes of cell growth and hormonal signaling among others (Ceuleers et al. 2016). However, the activity of such digestive proteases varies between individuals and are often insufficient in digesting proteins due to underlying problems that could be chronic or acute.

It has been reported that high protein intake can inhibit endogenous protease activity and result in incomplete digestion (Dallas et al. 2017). When such undigested proteins reach the colon, they are fermented by the colonic bacteria, which then produce toxic metabolites through putrefaction. This can further cause intestinal inflammation and other deleterious diseases such as colorectal cancer (Dallas et al. 2017; Kaur et a. 2017). Due to this, there has been an increase in the demand of external digestive protease enzyme supplements that can complement the functions of such naturally occurring proteases and consequently improve a consumer's digestive capacity and nutrient absorption (Oben et al. 2008). Several research studies have reported a positive effect of protease supplements on digestion (Craik et al. 2011; Pavan et al. 2012; Ianiro et al. 2016; Sousa et al. 2020). Previous studies have also suggested that protein intake accompanied with exogenous digestive protease is a better measure for protein digestion and absorption as compared to consuming protein hydrolysates (Jadhav et al. 2021). Intake of protein hydrolysates have also been reported to negatively affect the synthesis and secretion of pancreatic protease in digestive system (Kinouchi et al. 2012).

Various sources such as plants (papain and bromelain), animals (pancreatin), fungi (acid proteases from *Aspergillus sp.*) and bacteria (proteases from *Bacillus* sp.) have been explored for obtaining digestive protease enzymes (Ianiro et al. 2016; Pavan et al. 2012; Amri and Mamboya 2012; Minevich et al. 2015; Garvey et al. 2022; Razzaq et al. 2022). Though, currently commercial production and use of animal-derived proteases and microbial proteases is well established, the development of plant-derived proteases is warranted and could be of great promise. Recently, there has been a growing interest in using plant proteases due to their high stability under extreme conditions, wide substrate specificity and high activity under wide range of temperature and pH (Ravee et al. 2018). Additionally, it offers better safety since they are extracted from plant sources which are harmless and pose lesser threat as compared to their animal, fungal and microbial counterparts (Ianiro et al. 2016; Martinez et al. 2019). Taking the above crucial factors into consideration, the present study evaluated the effect of a plant protease based

proprietary formulation- 'MDZenPro' on protein digestion under *in-vitro* simulated gastrointestinal conditions.

Materials and Methods

Materials

Raw whey protein (WPC), raw whey protein isolate (WPI) and plant protein (PP) samples were obtained from Zywie Ventures Pvt Ltd. MDZenPro was supplied by Zenherb Labs Pvt Ltd. Pepsin, pancreatin, serine, o-phthalaldehyde (OPA), 1,4-Dithiothreitol (DTT) and other analytical grade chemicals used for preparing simulated gastric fluid and simulated intestinal fluid were procured from Sigma Aldrich, India.

Gastrointestinal Protein Digestion using INFOGEST Method

WPC, WPI and PP samples were subjected to simulated GI digestion based on the previously described INFOGEST method (Brodkorb et al. 2019; Jadhav et al. 2021). The first stage consisted of the simulated gastric protein digestion process. In this, 5 mL of protein sample of 50 mg/mL concentration was added to 3.9 mL simulated gastric fluid and 5 µL of 0.3 mol/L $CaCl_2$ and, the pH was adjusted to 3. This was followed by addition of pepsin to the solution such that its final concentration in the solution was 200 U/mL. Next, distilled water was added to adjust the volume to 10 mL. The solution was incubated at 37° C for 2 h under shaking conditions at 150 rpm. The samples were analyzed after incubation. The samples thus obtained were used as 'gastric digested sample'. Further, 7 mL of simulated intestinal fluid and 40 µL of 0.3 mol/L $CaCl_2$ were added to these digested samples and the pH was adjusted to 7. Pancreatin suspension was added such that its final concentration in the reaction mixture was 10 U/mL. The reaction mixture volume was adjusted to 20 mL using distilled water and further incubated for 2 h at 37° C in shaking incubator (150 rpm). The enzyme activity was stopped by placing the solution in a boiling water bath for 5 min. This reaction mixture was further centrifuged and, the supernatant obtained was used as 'gastro-intestinal digested sample'. Gastric and GI digested samples were also obtained using MDZenPro as an enzyme source in the above-mentioned method. Additionally, WPC was subjected to simulated GI digestion using a commercially available protease. The control sample was obtained without adding any enzyme. These samples were further analyzed for determining the degree of hydrolysis. The profile of the digested products was studied using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Degree of Hydrolysis

OPA reagent (175 µL) was added to 25 µL of appropriately diluted sample (25 mg/mL) in a microtiter plate and, the reaction mixture was incubated at 27 ± 2° C for 2 min. The absorbance of the solution was recorded at 340 nm wavelength. Standard curve was plotted using serine (3.125–100 µg/mL). The slope of the standard curve was analyzed for determining the serine equivalent free amino groups in the sample. The degree of hydrolysis was determined using the formula given below:

$$\text{Degree of hydrolysis (\%)} = \frac{\text{Free amino groups in the sample}}{\text{Free amino groups in the acid hydrolyzed sample}} \times 100$$

(The acid hydrolyzed sample was prepared by hydrolyzing the protein samples with acid. The total free amino group present in this hydrolyzed sample was determined by OPA assay.)

SDS-PAGE of In-vitro Digested Protein Samples

The digested protein products obtained from *in-vitro* digestion of whey and plant proteins using gastric enzymes and external enzymes along with the control were run on 10% SDS-PAGE. The gel was stained using Coomassie Brilliant Blue to analyze the presence of digested protein bands.

Results and Discussion

Analysis of Degree of Hydrolysis by OPA Method

MDZenPro was studied to determine its ability to augment the *in-vitro* gastric and GI digestion of whey protein and plant protein samples. Degree of hydrolysis for WPC, WPI and PP under simulated GI conditions were found to be 8.3%, 14.4% and 6.6% respectively. It was observed that the degree of hydrolysis of WPC, WPI and plant proteins that were treated with the MDZenPro was higher as compared to the protein samples that were not treated with the enzymes (Figure 1 and Figure 2). The untreated set showed a protein digestion of less than 10%. Hence, when compared to an untreated set, the set with MDZenPro showed approximately 59% faster digestibility. Protein sample WPI treated with MDZenPro showed maximum degree of hydrolysis after both gastric digestion (15.77%) and GI digestion (57.75%). Whey protein is one of the most popular protein supplements available in the market owing to its amino acid profile. In the present study, the degree of hydrolysis of WPC samples after treatment with a commercially available protease and MDZenPro under simulated GI conditions were found to be comparable, thereby validating the high efficiency of MDZenPro (Table 1).

Fruits such as papaya, pineapple, figs and kiwifruit are known to be rich sources of proteases (papain, bromelain, ficin and actinidin respectively) that are able to breakdown 'hard to digest' proteins such as gluten, casein and gelatin and enhance upper GI tract protein digestion (Kaur et al. 2010; Rawski et al. 2018; Troncoso et al. 2022). Research studies have described *in-vitro* static and dynamic models with different complexities for studying human digestion (Minekus et al., 2014, Kong and Singh, 2010, Kopf-Bolanz et al., 2012, Verwei et al., 2016). Lately, an updated and validated version of INFOGEST *in-vitro* static GI digestion simulation protocol has been widely employed for evaluating protein digestion (Sousa et al. 2020; Jadhav et al. 2021; Garvey et al. 2022). The procedure involves subjecting food samples to sequential gastric and intestinal digestion under standard laboratory setup while taking into consideration the physiological digestion parameters. INFOGEST is a simple method that possesses physiologic relevance of *in-vivo* food digestion (Brodkorb et al. 2019). However, to the best of our knowledge, limited work has been conducted for evaluating the effects of plant protease on protein samples under simulated GI digestion conditions based on the modified INFOGEST method. In the study conducted by Jadhav et al. (2021), plant protease-bromelain could not assist the digestion of whey protein under simulated digestion conditions. The lack of augmentation in GI protein digestion was attributed to its low stability under GI environment. However, our current findings found that the unique plant protease formulation of MDZenPro could effectively assist both gastric and GI digestion of whey protein thus, highlighting its promising commercial application.

Analysis of Protein Hydrolysis using SDS-PAGE

The profile of undigested and digested proteins (WPC, WPI and plant protein) were studied using SDS-PAGE. The SDS-PAGE profile pattern confirmed that upon simulated gastric and GI digestion, the protein samples treated with MDZenPro were hydrolysed into fragmented peptides (Figure 3). The WPC protein profiles obtained after hydrolysis with MDZenPro and commercial protease were comparable (Figure 4). These peptides possessed lower molecular weight as compared to the undigested protein samples (Figure 3 and Figure 4). The fragmentation of the protein samples after treatment with exogenous MDZenPro product further substantiates the digestion of protein samples.

Conclusion

The results of the degree of hydrolysis and electrophoretic analysis in the present study indicate that MDZenPro has a positive effect on protein digestion under simulated GI conditions. The unique feature is the presence of plant protease which provides a distinct advantage to the MDZenPro formulation since, it is a component derived from naturally safe sources unlike its animal and microbial counterparts. Thus, MDZenPro possesses a promising commercial application as a digestive aid for augmenting the breakdown of proteins.

Authors Contributions

Mr. Mihir Gadani contributed towards the concept designing for the ingredient. Ms. Ratna Upadhyay was involved in the development of the ingredient. Dr. Supriya Raut did the product formulation using the ingredient.

Acknowledgement

The authors would like to acknowledge Skanda life sciences for their contribution in conducting the study. We further extend our gratitude to Dr. Shruti Kakodkar for her contribution towards drafting of the manuscript. We also thank Ms. Sneha Badak for coordinating to get the manuscript ready and uploading the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Table 1: Percentage degree of hydrolysis of raw whey protein, whey protein isolate and plant protein samples following gastrointestinal digestion under simulated conditions

Protein Samples	**Mean Degree of Hydrolysis (%)**	
	Gastric Digestion	**Gastro-Intestinal Digestion**
1) Raw whey protein (WPC)		
WPC	8.30 ± 1.05	52.80 ± 4.29

WPC with MDZenPro	10.33 ± 1.06	56.20 ± 1.82
WPC with commercial protease	12.02 ± 0.66	58.83 ± 3.56
2) Whey protein isolate (WPI)		
WPI	14.4 ± 1.21	54.21 ± 1.17
WPI with MDZenPro	15.77 ± 1.18	57.75 ± 3.55
3) Plant Protein (PP)		
Plant Protein	6.60 ± 0.68	43.30 ± 4.02
Plant Protein with MDZenPro	8.0 ± 1.60	47.82 ± 2.93

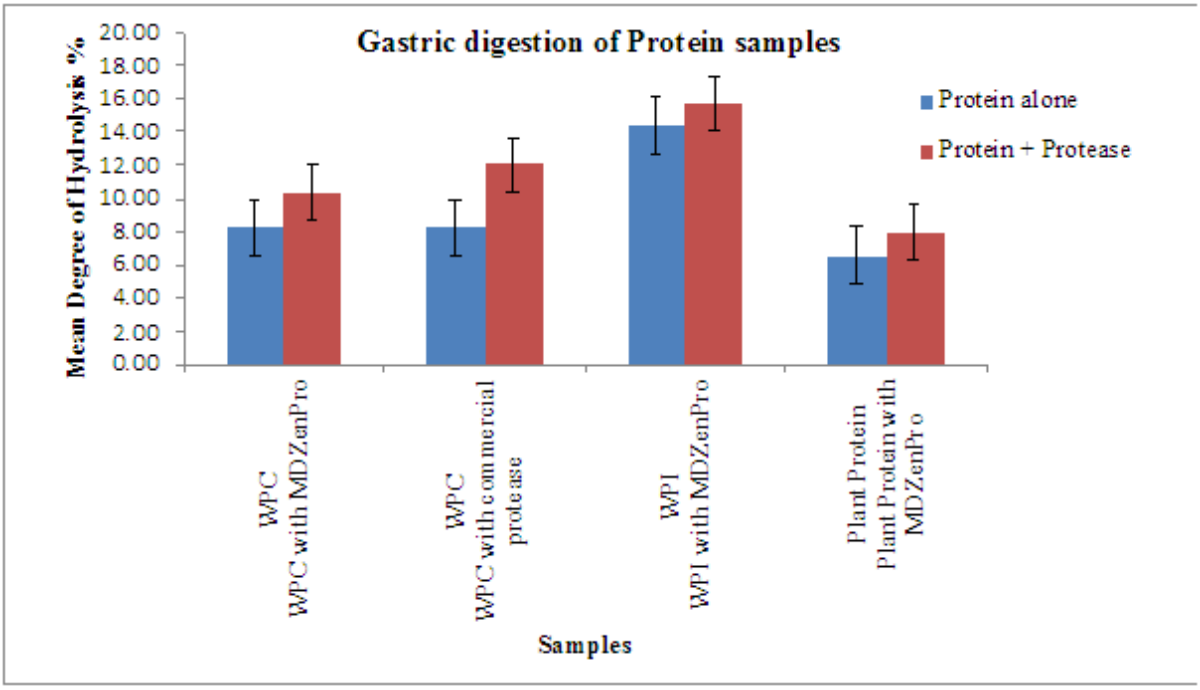

Figure 1: Degree of hydrolysis (%) of protein samples (WPC, WPI and Plant Protein) after gastric digestion with MDZenPro

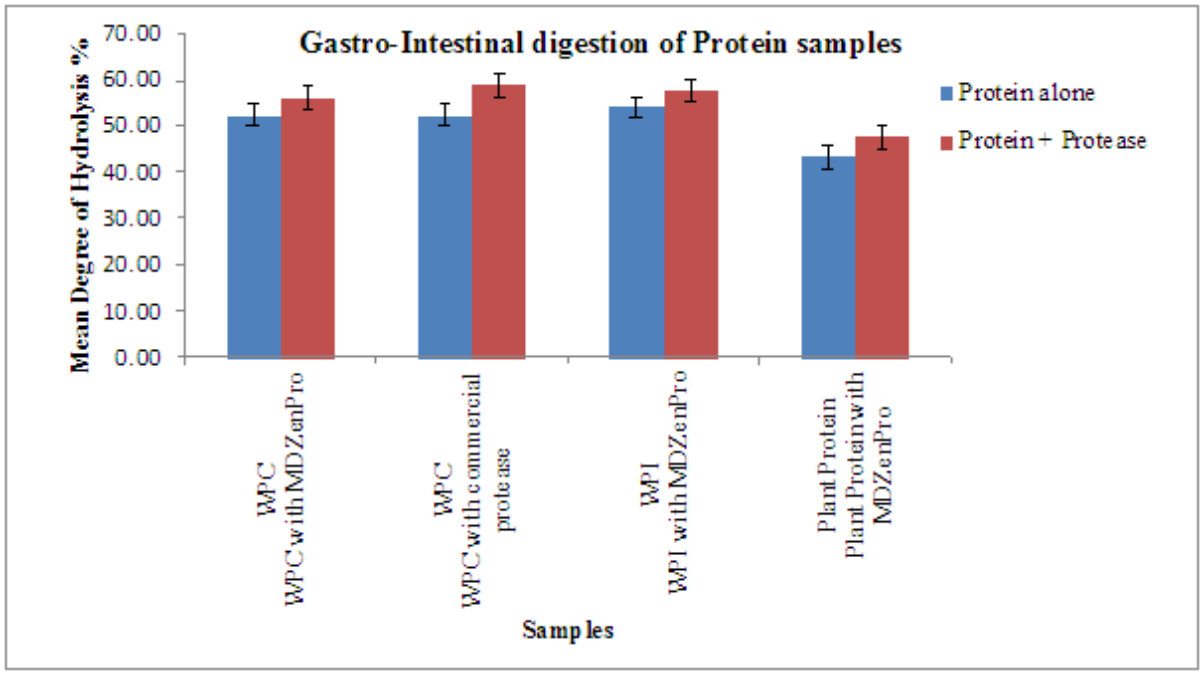

Figure 2: Degree of hydrolysis (%) of protein samples (WPC, WPI and Plant Protein) after gastrointestinal digestion with MDZenPro

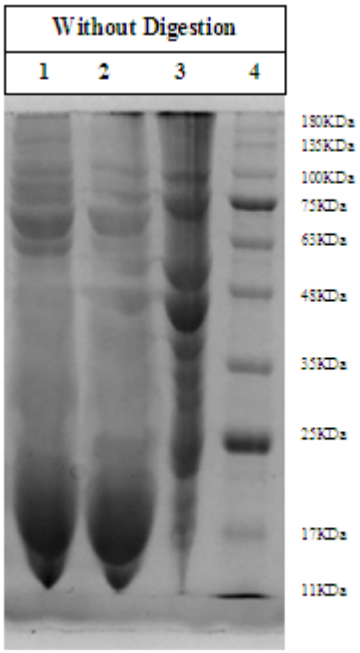

Figure 3: SDS-PAGE profile of undigested protein samples (WPC, WPI and plant protein). Lane 1 - WPC, Lane 2 - WPI, Lane 3 - Plant Protein, Lane 4 - Ladder

E-ISSN: 2582–2160, Volume: 4, Issue: 4, July-August 2022

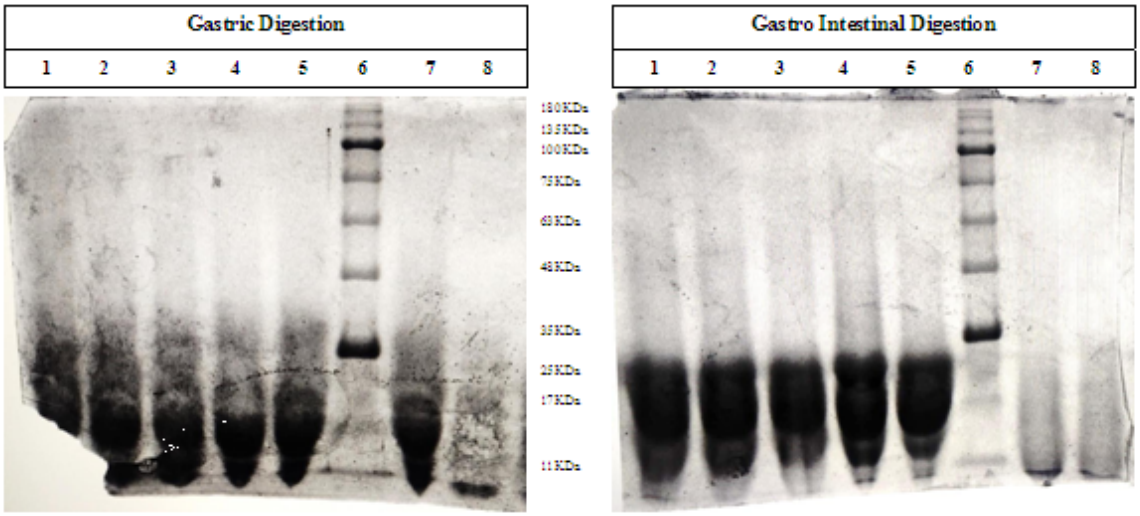

Figure 4: SDS-PAGE profile of protein samples subjected to simulated gastric and GI digestion (in absence and presence of exogenous enzymes *viz.* Commercial protease and MDZenPro) Lane 1 - WPC, Lane 2 - WPC + MDZenPro, Lane 3 - WPC + Commercial protease, Lane 4 - WPI, Lane 5 - WPI + MDZenPro, Lane 6 - Ladder, Lane 7 - Plant Protein, Lane 8 - Plant Protein + MDZenPro