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Effect of goat milk protein hydrolysates on antidiabetic activity and its insulin resistance in HepG2 cells

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ABSTRACT

Goat milk protein hydrolysates (Acid casein, Rennet casein, and Total milk protein) were prepared using different proteolytic enzymes (pancreatin, pepsin, and trypsin) throughout 8 hrs of hydrolysis time. The degree of hydrolysis (DH) of goat milk protein hydrolysate was estimated by *O*-Phthaldialdehyde (*O*PA) method at different hydrolysis times (0, 1, 2, 4, 6, and 8 hrs). The effect of different three types of goat milk protein hydrolysates on antidiabetic activity was investigated by testing the inhibition of *α*-amylase activity and glucose consumption rate in HepG2 cells. The types of goat milk protein hydrolysates, proteolytic enzymes, and hydrolysis time had a significant effect on DH. The highest DH was in rennet casein hydrolysates treated with pancreatin enzyme at 8 hrs, followed by acid casein hydrolysates treated with pancreatin enzyme at 6 and 8 hrs. While TMP hydrolysates treated with pepsin enzyme had the highest DH at 4 hrs. The highest hydrolysis time was at 2, 4, and 8 hrs by trypsin, pepsin, and pancreatin enzymes, respectively. The rennet casein hydrolysates by the pancreatin enzyme had the highest anti-diabetic impact of exhibiting its action by inhibiting the enzymes which have the main role in carbohydrate metabolism as the *α*-amylase enzyme and dramatically increased the glucose consumption rate in insulin-resistant HepG2 cells, followed by TMP hydrolysate treated with pepsin enzyme.

Keywords: Goat milk protein hydrolysates, Proteolytic enzymes, Diabetes, *α*-amylase activity, Insulin resistance, HepG2 cells.

1. Introduction

Goat milk is an important part of human nutrition because of its high nutritional value, hypo-allergenicity, and easy digestibility (Feng *et al.,* 2019). Goat milk has been demonstrated to be an excellent source of protein, calcium, potassium, phosphorus, niacin, pantothenic acid, riboflavin, thiamin, and vitamin A in the human diet (Liu and Zhang, 2022). Other milk components, such as proteins and their hydrolyzed products, have been also linked to provide multiple potential health values, such as anti-inflammatory, anti-diabetic, antihypertensive prevent cardiovascular disease, strengthen bones, boost immunity, and improve metabolism (Hammam *et al.,* 2022). Goat's milk can be used to produce a wide diversity of products, such as beverages, fermented products (cheese, buttermilk, yogurt, and frozen yogurt), ice cream, and butter (Aryana and Olson., 2017; Fazilah *et al.,* 2018). Furthermore, interest in goat milk is not only because of accepting goat milk as a food source but also due to the medical, nutritional, biological, and immunological applications and these properties support the processing and easiness commercialization of goat dairy products (Verruck *et al.,* 2019).
Nutraceutical food may provide both physical and mental benefits commonly attributed to the active components of the food. Recently, functional food is considered one of the foods that are required to be marketed to a large group of people worldwide (Siró *et al.,* 2008; Viuda-Martos, *et al.,* 2010). Functional food according to the consensus document issued by the european concerted

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action on science of functional foods is a portion of food that proves it positively influences one or more biological functions in the human body, improving the state of health and wellness, and reducing the risk for developing diseases (Diplock *et al*., 1999; Minervini *et al*., 2009)

Approximately 400 million people around the world live with type 2 diabetes mellitus, a chronic disease that causes almost 5 million deaths each year, which expected that this number will reach 640 million by 2040 (Cheng *et al*., 2019). Complications of diabetes comprise retinopathy, nephropathy, and cardiovascular disease, and they pose serious risks for people who suffer from type 2 diabetes (Parsamanesh *et al*., 2018). Notably, type 2 diabetes (T2D), which is characterized by insulin resistance and relative insulin insufficiency, accounted for around 90% of all diabetes cases (Czech, 2017; Saeedi *et al*., 2019). Therefore, improving insulin resistance and maintaining glucose homeostasis is currently the goal for effective approaches in the management of T2D (Liu *et al*., 2021). Particularly, the ingestion of proteins rich in essential amino acids or fast-absorbable proteins has resulted in a greater postprandial insulin secretion than the ingestion of a 'slow' protein (Tessari *et al*., 2007). Moreover, co-ingestion of protein hydrolysate (PH) in addition to carbohydrates has been shown to stimulate post-prandial insulin secretion and reduce postprandial plasma glucose increments in healthy volunteers and patients with type 2 diabetes (Geerts *et al*., 2011).

The liver is the principal organ for insulin action it maintains systemic glucose homeostasis with a balance between glycogenesis (glucose storage) and gluconeogenesis (glucose output) Under normal circumstances, will insulin binds to receptors in the liver to elevate glucose consumption and glycogenesis to maintain levels glucose in the blood (Kubota *et al.,* 2016).

Insulin resistance in the liver is characterized by insensitivity to insulin, resulting in hyperglycemia and hyperinsulinemia (Boonloh *et al.*, 2015), when insulin sensitivity is impaired, insulin resistance occurs and ultimately leads to a disruption of energy homeostasis and type 2 diabetes (Rungapamestry *et al.*, 2012; Milburn and Lawton, 2013). Consequently, this study aimed to evaluate the effects of the bioactive components of functional goat milk protein hydrolysates on glucose metabolism in insulin-resistant HepG2 cells and to enhance the prevention and improving insulin resistance.

2. Materials and Methods

2.1. Materials

Goat milk was purchased from EL-Serw, Animal Production Research Station Damietta, Ministry of Agriculture and Land Reclamation, Egypt. And used for preparing the milk protein hydrolysates. Pepsin 1:3000 U/g: (0.8 Anson units/mg). and Trypsin 2000 U/g: (0.2 Anson units/mg) were Purchased from Qualikems Fine Chemicals PVT.LTD., India. Pancreatin: (EX pancrease 0.197 mg/g) was purchased from LOBA Chemie, PVT.LTD, Mumbai, and Maharashtra, India. Starch (Potato starch), Sodium Potassium tartrate, and 3,5 dinitro salicylic acid were obtained from Oxford Lab. Chemicals. India. Sodium Hydroxide (NaOH), sodium acetate, sodium tetra borate, and methanol alcohol were obtained from MRM Chemicals Egyptian Company. *α*- amylase (1:2000 U/g): was purchased from oxford lab Chemise, India. Rennet: Calf rennet powder (Ha-La) with the strength of (10N) was obtained from CHR- Hansen's Lab. Denmark. Glucose kit assay was purchased from Chemicals Egyptian Company and Mixtard 30 insulin Human produced by Novo Nordisk in Denmark it's Suitable for type 2 diabetes. - *O*-phthaldialdehyde (*O*PA), β-mercaptoethanol, and Sodium dodecyl sulfate (SDS) were purchased from Sigma Aldrich Co., Saint Louis, USA.

2.2. Methods

2.2.1. Chemical composition of goat's milk

The chemical composition of goat milk was determined by Milk Analyzer Master SN: 21768, made in England.

2.2.2. Preparation of different types of goat milk protein

Acid casein, rennet casein, and total milk proteinases (casein and undenatured whey protein) were prepared from goat skim milk following the method of Morr, (1985).

2.2.3. Preparation of goat protein hydrolysates by different proteolysis enzymes

Goat's milk protein hydrolysates were prepared with the method of different proteolysis enzymes such as pancreatin, pepsin, and trypsin according to the method (Otte *et al*., 2007; Assem *et al.,* 2017) Protein solution, (2% w/w) on a protein basis with little modification at different hydrolysis times (0, 1, 2, 4, 6, and 8hrs). The condition of hydrolysis described by Esan and Fasasi, (2013) procedures are shown in Table (1).

Table 1: Conditions employed for hydrolysis of goat milk proteins.

Enzyme	pH	E/S (w/w)	Temperature (°C)
Pepsin	2.0	1:100	37°
Trypsin	8.0	1:100	40°
Pancreatin	8.0	1:100	40°

E/S: Enzyme/ Substrate ratio, w/w: weight/weight.

2.4. Determination of the degree of hydrolysis of goat milk proteins

The degree of hydrolysis (DH) of goat protein hydrolysates was determined by reacting free amino acids with *O*-Phthaldialdehyde (*O*PA), according to Luo *et al*. (2014). The absorbance of the mixture was measured at 340 nm using a spectrophotometer (Jenway® Genova Life Science Spectrophotometer UV/Visible made in the U.S.A).

2.5. *In vitro* antidiabetic activity of goat milk protein hydrolysates by different proteolysis enzymes

2.5.1. Inhibition of *α*-amylase enzyme activity

Goat milk protein hydrolysates were subjected to antidiabetic activity assessment according to the methods described by Manikandan *et al*. (2013). The assessment inhibition of *α*-amylase enzyme was an excellent strategy to screen extracts for antidiabetic activity. Thus, the inhibition of *α*-amylase activity was determined spectrophotometrically (Jenway® Genova Life Science Spectrophotometer UV/Visible made in the U.S.A) according to the method described previously by Malik and Singh, (1980). For enzymatic reaction, 1 ml of goat's protein hydrolysates was mixed with an equal volume of starch solution and left to react with a *α*-amylase solution for 3 min at 25ºC. The generation of maltose was quantified by the reduction of 3, 5 dinitro salicylic acid to 3- amino-5- nitro salicylic acid. The reaction was detected at 540 nm by spectrophotometer (Jenway® Genova Life Science Spectrophotometer UV/Visible made in the U.S.A).

2.5.2. Effect of goat milk protein hydrolysates on glucose consumption rate in insulin-resistant HepG2 cells

The HepG2 cells (1×10^5 cells/mL) were incubated at 37°C in a humidified atmosphere that contained 5% CO_2. The cells were treated with 5.5 µl glucose (5% v/v) to represent normoglycemia for 24 h, followed by 100 µl insulin (5% v/v) stimulation for 10 min. It established a model of high-glucose-induced insulin resistance as mentioned previously (Cordero-Herrera *et al*., 2014; Song *et al.,* 2017). Briefly, the HepG2 cells were cultured in a medium supplemented with 30 µl (5% v/v) glucose to represent hyperglycemia with or without goat milk protein hydrolysates for 24 h, followed by 100 µl insulin (5% v/v) stimulation for 10 min, and then harvested.

2.5.3. Glucose Consumption Rate (GCR)

Determination of the glucose consumption rate as reported previously, with slight modifications (Chen *et al.,* 2016; Li *et al.,* 2017 and Gowd *et al.,* 2018). The HepG2 cells were seeded into 96-well plates at 1×10^5 cells/mL for 24 h; blank wells contained only medium. Then, 30 µl glucose (5% v/v) was added to the medium in the absence (model group) or presence (treatment group) of goat milk protein hydrolysates, followed by incubation for 24 h and 100 µl insulin (5% v/v) stimulation for 10 min; PBS was used as a control. Glucose concentration was measured using the glucose oxidase-peroxidase method with an assay kit based on a previous reference method by Wang *et al.* (2018) to

calculate glucose consumption, the amount of glucose left in the medium after incubation is subtracted from the initial amount.

2.6- Statistical analysis:

All experiments were carried out in triplicate, with an average of three repetitions being reported. The data were evaluated statistically using IBM ® SPSS® Statistics software version 19 and Duncan's Multiple Range Test was applied at the 0.05 level (Bryman and Cramer, 2012).

3. Results and Discussion

3.1. Chemical composition of goat's milk.

The chemical composition of goat milk was determined by Milk Analyzer Master it's shown in Table (2) goat milk had 3.5 % total protein and 3.9 % fat and was used throughout the study to obtain acid casein, rennet casein, and TMP.

Table 2: Chemical composition of goat's milk.

Components	Average
Protein %	3.5± 0.1
Fat %	3.9± 0.1
Lactose %	5.1± 0.1
Total solids %	12.5± 0.1
SNF %	8.63±0.1
Ash %	0.7± 0.1

Data are mean values ± SE of triplicate results.

3.2- Determination of the degree of hydrolysis of goat milk proteins

The degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. Several methods are used to determine DH including the *O*-phthaldialdehyde (*O*PA). The principle of the reaction is based on the response of *O*PA with primary amino groups and an SH compound (Dithiothreitol, DTT) to form a compound that will absorb light at 340 nm (Nielsen *et al*., 2001). The *O*PA spectrophotometric assay for measurement of proteolysis depends on the ability to measure released α-amino groups in the presence of protein, the average proteolytic.

Fig (1) illustrated the DH values of acid casein, rennet casein, and total milk proteins treated with different three proteolytic enzymes (pancreatin, pepsin, and trypsin) during 8 hrs of hydrolysis. There were significant differences in DH value among all treatments and time of hydrolysis, which was noticed with acid and rennet caseins treated with pancreatin enzyme.

While the highest significant DH value was in TMP treated with pepsin during 4 hrs of hydrolysis. On the other hand, The DH value decreased after 4 hrs of hydrolysis for acid and rennet caseins treated with pepsin or trypsin. DH values of acid and rennet caseins increased by increasing the time of hydrolysis for pancreatin treatments. These findings agree with the results of (Otte *et al.*, 2007).

Caseins are rich in proline, which adds to the inhibitory activity of peptides, and/or to the higher flexibility of the caseins making them more susceptible to proteolysis and results in a higher degree of hydrolysis.DH values for TMP treated with pepsin enzyme were the highest value till the end of hydrolysis time (Lacroix and Li-Chan 2012).The variation in time hydrolysis depends on goat milk protein types and enzyme treatment.

Silvestre *et al.* (2013) observed that production of non-protein nitrogen increased as incubation time progressed and that was shown all over the tested proteins with different enzymes but the degree of hydrolysis varied from enzymes, and equal degradation points of all enzymes were observed after 4 hours of the goat acid casein. The highest DH values were significantly different in rennet casein treated with pancreatin enzyme within 8 hrs of hydrolysis, on the contrary, the least significant DH value was noticed in rennet caseins treated with trypsin enzyme at 2 hrs.

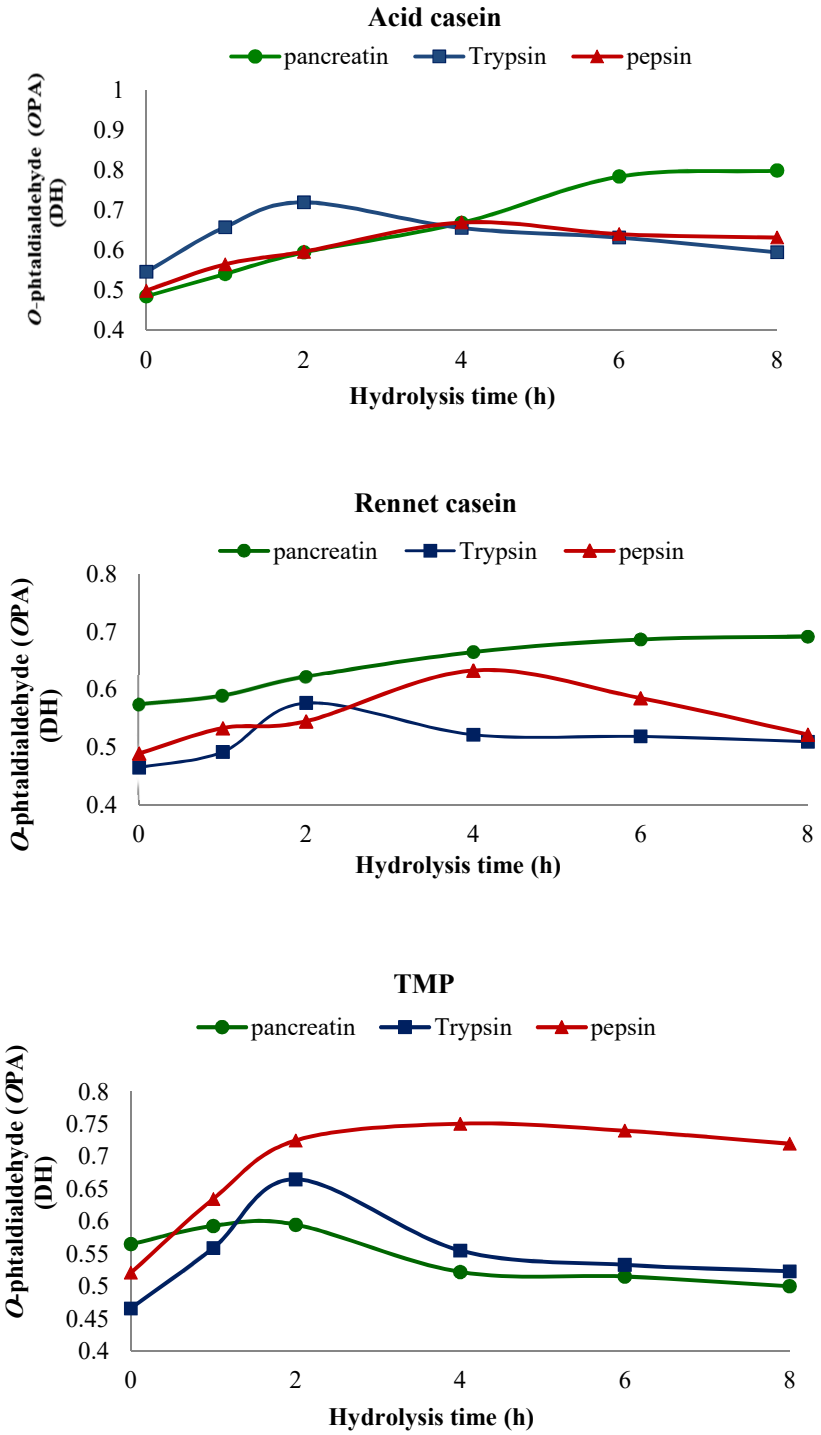

Fig. 1: Degree of hydrolysis (DH) of goat milk protein hydrolysates treated by different proteolytic enzymes

3.3- *In vitro* antidiabetic activity of different goat milk protein hydrolysates.

3.3.1- Inhibition of *α*-amylase enzyme activity.

The most beneficial treatment of type 2 diabetes is achieved by maintaining an optimal blood glucose level after a meal. The *α*-amylase enzyme breaks down long-chain carbohydrates to produce glucose in the small intestine. Inhibition of *α*-amylase activity is a key method to inhibit glucose production in the body (Jiang *et al.,* 2021).

Fig (2) showed the inhibition of *α*-amylase activity by using acid casein hydrolysates resulting from different proteolytic enzymes (pancreatic, pepsin, and trypsin) during 8 hrs of hydrolysis time. Inhibition of *α*-amylase activity significantly increased by increasing of hydrolysis time in acid casein treated with pancreatin enzyme while acid casein hydrolysate with pepsin inhibited of *α*-amylase activity till 4 hrs of hydrolysis time followed by a gradual decrease. Whereas, inhibition of *α*-amylase activity increased after 1 and 2 hrs of hydrolysis time followed by a significant decrease in acid casein treated with trypsin enzyme.

Due to release peptides that inhibit the action of *α*-amylase activity, the acid casein treated with pancreatin enzyme had the best results at the following times (4, 6, and 8 hours) with rates of 76.5, 85.2 and 90.3%, respectively, followed by the treatment with enzyme pepsin enzyme 70.7% at 4 hours, then trypsin enzyme 67.5% at 2 hours.

Awosika and Aluko, (2019) revealed that protein hydrolysis was conducted using different proteolytic enzymes, and goat protein hydrolysates containing different types of peptides to inhibit *α* -amylase activity.

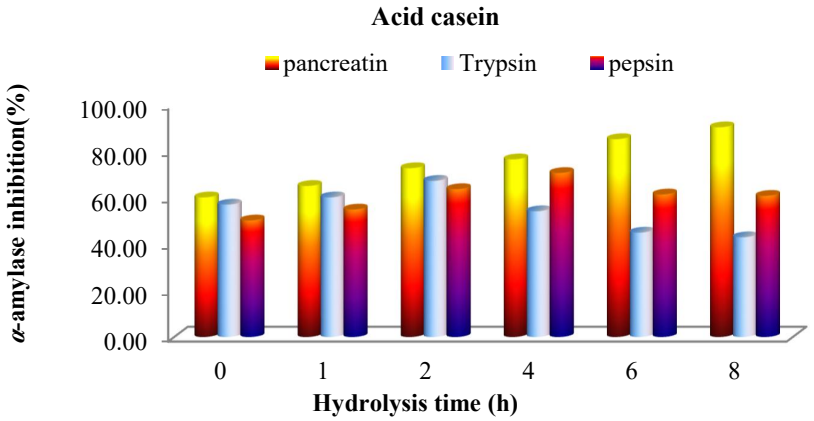

Fig. 2: Effect of goat acid casein hydrolysates on inhibition of *α*-amylase activity.

The data of Fig. (3) Revealed the inhibition of *α*-amylase activity by goat rennet casein hydrolysates with different proteolytic enzymes (pancreatic, pepsin, and trypsin) for 8 hrs of hydrolysis time. Inhibition of *α*-amylase activity occurred with an increase in the time of protein degradation due to the release of peptides that inhibit of *α*-amylase enzyme activity from goat milk protein hydrolysate. The highest significant inhibition of *α*-amylase was in rennet casein treated with pancreatin enzyme at the following times (4, 6, and 8 hrs), and the inhibition of *α*-amylase activity was 89.9, 92.7, and 95%, respectively, followed by rennet casein treated with trypsin enzyme (90% at 2 hours). While inhibition of *α*-amylase was high in rennet casein treated with pepsin (88.4% at 4 hrs) followed by a remarkable significant decrease till the end of hydrolysis time.

Arise *et al*. (2019) mentioned that amino acids present in milk bonded to one another in the primary structure of protein remain inactive, the enzymatic hydrolysis can break down this primary structure of protein releasing peptide sequences that can be used as therapeutic agents. Of particular importance are those peptides with multifunctional bioactivities. So related conditions like hypertension and diabetes have become major public health conditions. Thus, goat rennet casein hydrolysates can play a great role as a natural source of the antidiabetic agents.

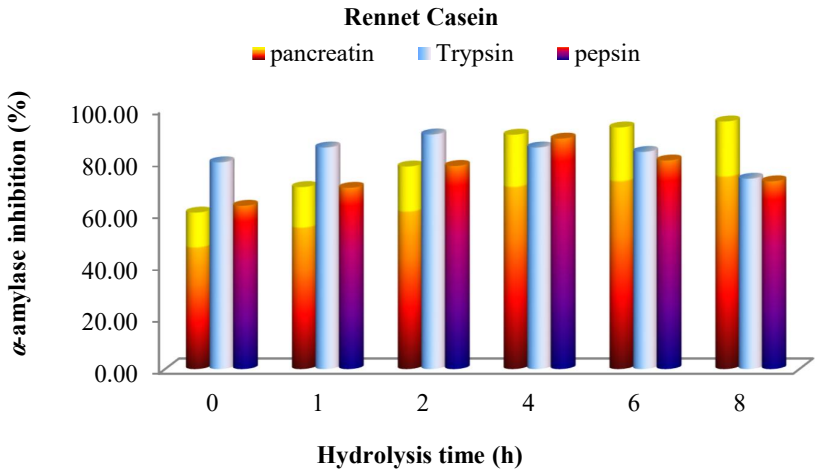

Fig. 3: Effect of goat rennet casein hydrolysates on inhibition of *α*-amylase activity.

Fig (4) showed the effect of TMP hydrolysates on the inhibition of *α*-amylase activity during 8 hrs of hydrolysis time. The high inhibition of *α*-amylase activity was 66% using goat TMP treated with pancreatin enzyme from the beginning till 1 and 2 hrs of hydrolysis time.

Furthermore, the effect of the pancreatin digest of TMP hydrolysates on the inhibition of *α*-amylase activity gradually decreased with increasing hydrolysis time for 4, 6, and 8 hrs. The effect of pepsin digest of TMP hydrolysates on inhibition of *α*-amylase activity gradually increased by increasing hydrolysis time till 4 hrs followed by a significant decrease.

Goat milk protein hydrolysates had a significant inhibitory effect on *α*-amylase activity and this effect of goat protein hydrolysates varied according to hydrolysis time and DH by pancreatin, pepsin, and trypsin enzymes treatment. Generally, inhibition of *α*-amylase activity was dependent on proteins hydrolysate types, and hydrolysis time. Diabetic-related enzymes can be inhibited by bioactive compounds, including dietary proteins, hydrolysates, and peptides derived from food-grade proteins (Oseguera-Toledo *et al.,* 2015).

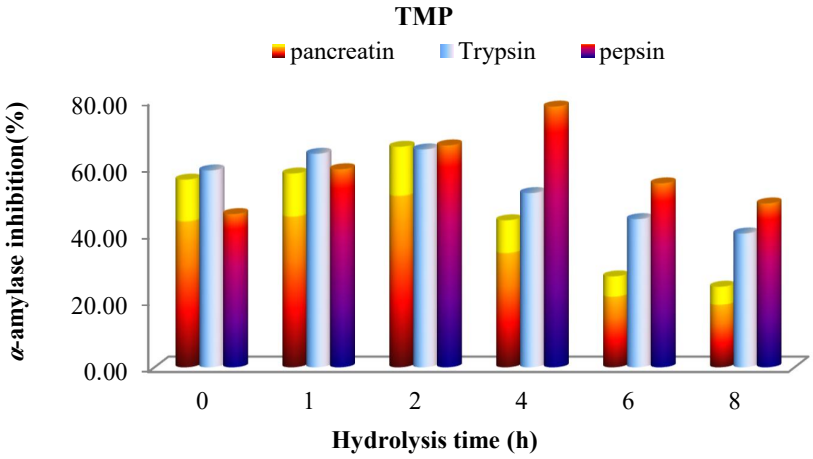

Fig. 4: Effect of goat TMP hydrolysates on inhibition of *α*-amylase activity.

3.3.2. Effect of goat milk protein hydrolysates on glucose consumption rate (GCR) % in insulin-resistant HepG2 cells.

Glucose consumption rate is one of the methods for measuring antidiabetic activity. The effect of goat milk protein hydrolysates produced by different proteolytic enzymes (pancreatin, pepsin, and trypsin) at different hydrolysis times (0. 1. 2. 4. 6, and 8 hrs) on glucose consumption rate % in HepG2 cells was studied.

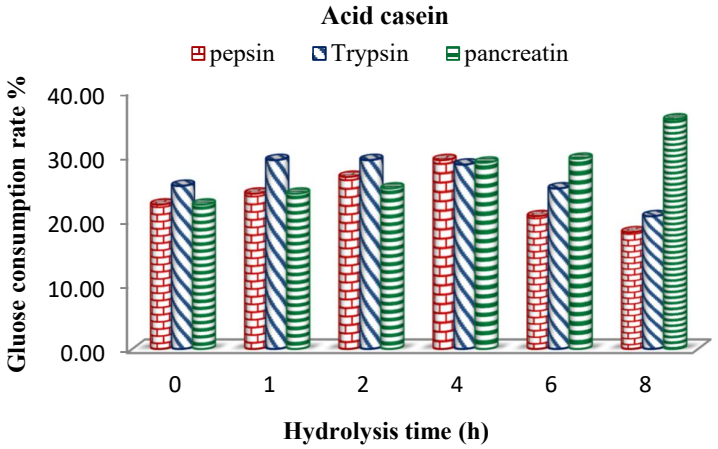

Fig. 5. Effect of goat acid casein hydrolysates on glucose consumption rate %

The data in Fig (5) revealed the occurrence of glucose consumption at a highly significant rate for goat acid casein hydrolysates by pancreatin enzyme after 4, 6, and 8 hrs of hydrolysis time. The glucose consumption rate % was significantly high in acid casein hydrolysates from pepsin and trypsin during the first 4 hrs of hydrolysis time. An increase of DH of acid casein hydrolysates by increasing hydrolysis time by pancreatin treatment had the highest significant effect on glucose consumption rate %. The DH with increasing hydrolysis time for 8 hrs by pancreatin enzyme treatment caused a release of insulin-like peptides and glucose consumption in cells (Gong *et al.,* 2020). By slight modification in this study.

The DH of goat acid casein hydrolysates gradually increased from the beginning of hydrolysis time to the end of the release of unique peptides similar in their action to insulin, which led to the consumption of glucose injected into the insulin-resistant HepG2 cells. The glucose consumption rate % increased from zero time of degradation to reach the best rates (33.8, 36.6, and 39.6%), respectively, at the following times of acid casein hydrolysates (4, 6, and 8 hours), then followed by the enzyme trypsin and pepsin treatments.

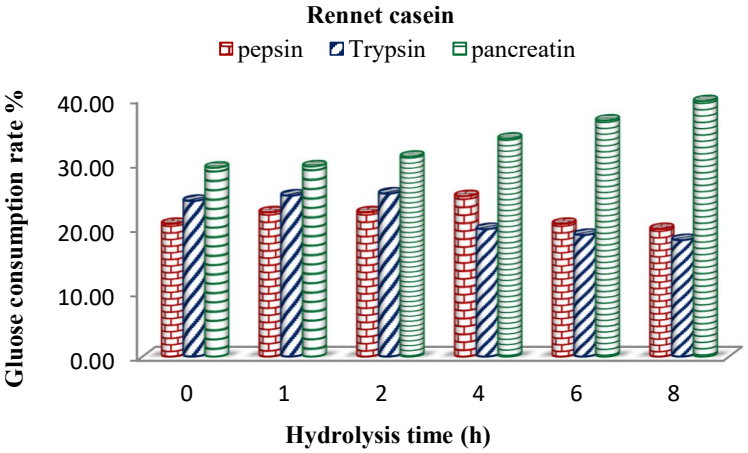

Fig. 6: Effect of goat rennet casein hydrolysates on glucose consumption rate %

Fig. (6) showed the decrease in the glucose consumption rate (GCR) of rennet casein hydrolysates resulting from pepsin and trypsin enzymes compared to rennet casein hydrolysates with pancreatin enzyme. While, the highest significant glucose consumption rate was in rennet casein hydrolysates using pancreatin enzyme at the following values 28.9, 29.5, and 30.6%, respectively at the following hydrolysis times 4, 6, and 8 hrs. These results correspond to Guo *et al.* (2020).

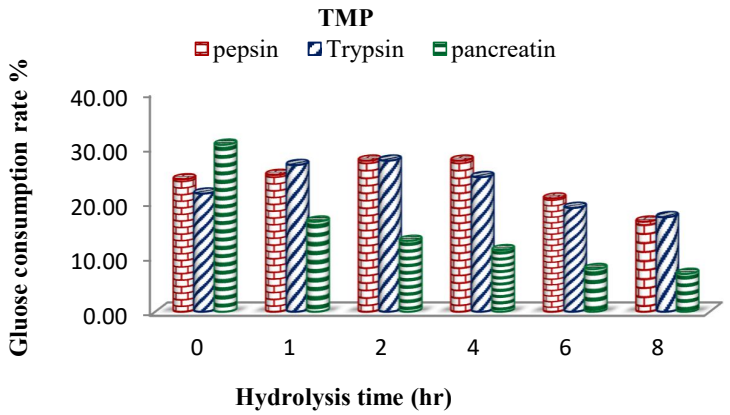

Fig. 7: Effect of goat TMP hydrolysate on glucose consumption rate %

Fig (7) illustrated the highest glucose consumption rate % was in total milk protein hydrolysates with pancreatin enzyme at the beginning of hydrolysis (30.6%), followed by goat TMP hydrolysates with pepsin enzyme. After 2 and 4 hrs of hydrolysis, GCR increased in goat TMP hydrolysates treated with pepsin and trypsin. While GCR in the insulin-resistant HepG2 cells significantly decreased with the increase of DH of hydrolysis time.

Results agreed with the data of the degree of hydrolysis estimated by the method (*O*PA) and the difference in glucose consumption rate % of insulin-resistant HepG2 cells with different proteolysis enzymes. The glucose consumption rate % in the insulin-resistant HepG2 cells is dependent on protein hydrolysate types, DH, and α-amylase activity. These results agreed with Gong *et al*. (2020).

4. Conclusion

The efficiency of goat milk protein hydrolysates produced by different proteolytic enzymes (pancreatin, pepsin, and trypsin) at different hydrolysis times was investigated by measuring the DH at different hydrolysis times, inhibition of α-amylase activity and the rate of glucose consumption in insulin-resistant HepG2 cells, which controlled blood glucose levels for Patients type2 diabetics.

Insulin-resistant HepG2 cells model is generally used to investigate the pathogenesis of insulin-resistance and evaluate the hypoglycemic effects of milk-derived bioactive substances *in vitro* experiments. The degree of hydrolysis of goat milk protein hydrolysates had a significant effect on α-amylase activity and glucose consumption rate % of the insulin-resistant HepG2 cells model. Furthermore, and goat rennet casein hydrolysates showed significant improvement in glucose metabolism by the inhibition of α-amylase activity and glucose consumption rate in insulin-resistant HepG2 cells model.

These findings indicated that bioactive components in goat rennet casein hydrolysates contributed to the inhibition of α-amylase activity and improved insulin-resistance in HepG2 cells.

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