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EXTRACTION AND CHARACTERIZATION OF CHICKEN PEPSIN WITH EVALUATION OF ITS MILK-CLOTTING ACTIVITY IN SKIM MILK

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KEYWORDS:
chicken pepsin, pepsin extraction, milk-clotting activity, skim milk

ABSTRACT

The development of poultry farming creates opportunities to obtain pepsin, a proteolytic enzyme with milk-clotting activity, from the chicken proventriculus (*Gallus gallus*). This organ, considered a by-product, can serve as a source of proteases, including milk-clotting enzymes that may be used as substitutes for conventional milk-clotting agents in the dairy industry. This study aims to extract pepsin from chicken proventriculus, characterize it, and evaluate its potential use as a rennet substitute. It also attempts to determine the optimal milk clotting conditions (enzyme volume, pH, temperature, and $CaCl_2$ concentration) based on milk flocculation and clotting times. According to the obtained results, the clarified extract of chicken pepsin showed a milk-clotting activity of 63.48 ± 0.21 RU/ml, a specific milk-clotting activity of 1.56 ± 0.19 (RU mg^{-1}), a clotting strength of 12957 ± 399 (SU), a protein content of 43.49 ± 0.75 mg/mL, and a proteolytic activity of 0.91 ± 0.140 µg/mL·h. The highest milk-clotting activity of chicken pepsin was observed between 38 °C and 40 °C, at pH 6.6 and 6.8, with an enzyme volume of 3 mL, and at a $CaCl_2$ concentration of 5 mM. The obtained results indicate the potential use of chicken pepsin as a substitute for rennet in milk clotting. It could also be used in other biological or industrial applications.

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ЭКСТРАКЦИЯ И ХАРАКТЕРИСТИКА КУРИНОГО ПЕПСИНА С ОЦЕНКОЙ ЕГО МОЛОКОСВЕРТЫВАЮЩЕЙ АКТИВНОСТИ В ОБЕЗЖИРЕННОМ МОЛОКЕ

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КЛЮЧЕВЫЕ СЛОВА:
куриный пепсин, экстракция пепсина, молокосвертывающая активность, обезжиренное молоко

АННОТАЦИЯ

Развитие птицеводства открывает возможности для получения пепсина, протеолитического фермента с молокосвертывающей активностью, из железистого желудка кур (*Gallus gallus*). Этот орган, считающийся побочным продуктом производства, может служить источником протеаз, в том числе молокосвертывающих ферментов, которые могут использоваться в качестве заменителей традиционных коагулянтов в молочной промышленности. Цель данного исследования — извлечь пепсин из железистого желудка кур, охарактеризовать его и оценить его потенциальное использование в качестве заменителя сычужного фермента. Также предпринята попытка определить оптимальные условия коагуляции молока (объем фермента, pH, температура и концентрация $CaCl_2$) на основе времени флокуляции и коагуляции молока. Согласно полученным результатам, осветленный экстракт куриного пепсина показал молокосвертывающую активность 63.48 ± 0.21 RU/мл, удельную молокосвертывающую активность 1.56 ± 0.19 (RU $мг^{-1}$), прочность геля 12957 ± 399 (SU), содержание белка составляло 43,49 ± 0,75 мг/мл, а протеолитическая активность — 0,91 ± 0,140 мкг/мл·ч. Наибольшая молокосвертывающая активность куриного пепсина наблюдалась в диапазоне температур от 38 °C до 40 °C, при pH 6.6 и 6.8, объеме фермента 3 мл и концентрации $CaCl_2$ 5 мМ. Полученные результаты указывают на потенциальную возможность использования куриного пепсина в качестве заменителя сычужного фермента при коагуляции молока, а также в других биологических или промышленных целях.

1. Introduction

Proteases are among the most widely used commercial enzymes due to their multiple applications in various fields [1]. Protease sales alone account for over 60% of total enzyme sales [2]. These enzymes play an important role in the pharmaceutical and food industries, as well as in industrial biotechnology. The food industry is the largest consumer of proteases [3]. Within this industry, one of the major applications is the use of rennet in cheesemaking [4].

Cheesemaking involves several steps, one of the most fundamental of which is milk clotting. Clotting is traditionally achieved by the action of rennet, which is industrially extracted from the abomasum of unweaned calves [5,6]. Until the 1950s, rennet was the dominant enzyme used in cheesemaking [7]. However, its availability declined because its production depends on the meat industry. Currently, traditional animal rennet production covers only 20–30% of the coagulant demand [8].

The shortage of rennet on the global market has led to an increase in its price. At the same time, cheese production has gradually increased over the years. This situation has prompted the search for alternative enzyme preparations of various origins that can coagulate milk in a similar way to rennet [9,10]. In the search for rennet substitutes, microbial

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proteases, particularly those produced by genetically modified microorganisms, have yielded satisfactory results [11]. However, in recent years, natural extracts have attracted increasing attention as an alternative [12]. They have high proteolytic activity and are generally active over a wide range of temperatures and pH [13].

Among the substitutes, chicken pepsin (EC 3.4.23.1) is a protease with milk-clotting activity [14]. Chicken pepsin is an enzyme extracted from chicken proventriculi (*Gallus gallus*), which is considered a by-product of slaughter. These organs, averaging 3 cm in length, are located above the gizzard and covered with a layer of epithelium formed of columnar cells. These cells are responsible for the secretion of pepsinogen and hydrochloric acid. This by-product could be valorized because of the ease of pepsin extraction, its increasing availability, associated with the development of poultry farming, and its potential direct use, particularly in cheesemaking, as a means of addressing rennet shortages.

Extensive research has been conducted on the effect of chicken pepsin on milk clotting. However, limited information is available on the effects of pepsin on proteolysis and the sensory characteristics of cheeses. Several factors, including enzyme volume, temperature, pH, and Ca^{2+} concentration, influence enzyme-induced milk clotting [13]. This study aims to optimize clotting conditions using clotting times of skim milk treated with chicken pepsin as optimization criteria.

2. Objects and methods

2.1. Berridge substrate

The Berridge substrate is obtained by dissolving skim milk powder at 12 % (w/v) in 0.01M $CaCl_2$ solution. To prevent microbial growth, sodium azide is added at 0.04 % (w/v). The milk is then stored at 4 °C for at least 12 hours to ensure complete rehydration and stabilization of the casein micelles.

2.2. Chicken pepsin extract

After the chickens were slaughtered and eviscerated, approximately 10 kg of proventriculi were transported to the laboratory under refrigerated conditions. The proventriculi were trimmed of fat, rinsed under running water, and drained. Pepsin extraction was carried out according to [15] by grinding 100 g of proventriculi in 300 mL of a solution containing 30 g/L NaCl and 7g/L $NaHCO_3$, and stirring the mixture for 3 hours at 4 °C. After filtering, the crude extract was acidified with 3M HCl to pH 2 to activate the pepsin, and the mixture was incubated for 30 min. The pH was subsequently adjusted to 6.6 using 1M NaO H.

2.3. Physicochemical analysis of skim milk

Milk pH was measured at 25 °C using a pH meter (HI2210, Hanna Instruments, USA; resolution: 0.01 pH units), calibrated with buffer solutions prior to use. Titratable acidity was determined by titrating milk with 0.1M NaOH in the presence of phenolphthalein as an indicator. Milk density was measured using a lactodensimeter (acc. Gerber, large T20 °C, Funke Gerber, Germany). The concentrations of mineral elements were determined by atomic absorption spectrometry (DW-220A/B Monochrome Czerny-Turner, Chongqing Drawell Instrument, China) (ISO 6869:2000[1])

Total solids content was determined by drying at 103 ± 2 °C for 3 hours (ISO 6731:2010[2]). Nitrogen content was determined by the Kjeldahl method (ISO 8968-1: 2014[3]), and converted to protein content according to the following equation: $Mp = MN \times 6.25$ (*Mp*: protein mass, *MN*: nitrogen mass). Fat content was determined according to the Gerber method (ISO 19662:2018[4]), and lactose content was determined by the Bertrand method (ISO 26462:2010[5]).

2.4. Determination of optimal clotting conditions

To better characterize the milk-clotting properties of chicken pepsin, we studied the variation of clotting time as a function of pH, temperature, and $CaCl_2$ concentration. The shortest flocculation time recorded for each parameter indicated greater milk-clotting activity. The pH, temperature, and $CaCl_2$ concentration values were determined as follows:

The pH was adjusted to 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, and 6.8 with HCl (0.1M) or NaOH (0.1M) solutions in tubes, each containing 10 mL of Berridge substrate of the same preparation at 30 °C in a water bath (Memmert GmbH + Co. KG, Germany). One mL of each enzymatic dilution (chicken pepsin) was added, and the clotting time was determined [7].

To assess the effect of temperature, tubes containing 10 mL of Berridge substrate at pH 6.6 were incubated in the water bath (Memmert GmbH + Co. KG, Germany) at temperatures ranging from 28 °C to 40 °C (2 °C intervals). After 5 minutes of incubation, 1 mL of each enzyme dilution (chicken pepsin) was added, and the clotting time was recorded.

The influence of milk $CaCl_2$ concentration on the milk-clotting activity of the enzyme extract was studied at 35 °C and pH 6.5, using the Berridge substrate prepared with $CaCl_2$ concentrations ranging from 5 mM to 35 mM (range of 5 mM intervals). The effect of enzyme volume on the proteolytic activity of the enzymatic extract of pepsin was determined according to [16] by varying the enzymatic volume from 1 to 7 mL.

2.5. Characterization of the enzymatic extract

One mL of diluted chicken pepsin solution was added to 10 mL of prewarmed Berridge substrate in assay tubes at 30 °C. The clotting time was recorded as the point at which the first visible flakes appeared on the surface of the tilted tube. Milk-clotting activity was expressed in Rennet Units (RU), where one RU is defined as the amount of enzyme in 1 mL that coagulates 10 mL of standard substrate in 100 seconds at 30 °C [4], according to the following equation:

$$\text{RU (mL}^{-1}) = (10 \times V)/(T \times v), \quad (1)$$

where RU: Rennet unit, *V*: milk volume, *v*: enzyme volume, *T*: clotting time (sec).

Protein content of the enzyme extract was determined using the same method as described for milk proteins, with the conversion factor of 6.25. Clotting strength is defined as the volume of milk coagulated per unit volume of enzyme extract, within 40 min, at 35 °C and pH 6.4. Specific activity was calculated directly as the number of units of activity per unit mass (RU/mg) of protein. This parameter reflects the degree of enzyme purity [12].

Proteolytic activity was determined according to [17]. This assay measures the rate of casein degradation by the enzyme during the primary (enzymatic) phase. For this purpose, the concentration of casein hydrolysis products soluble in trichloroacetic acid (TCA) at a final concentration of 12 % was measured.

2.6. Statistical methodology

Data analysis was carried out using a one-way analysis of variance (ANOVA) to evaluate the effect of different levels of a single independent factor (e.g., pH, temperature, concentration, etc.) on a dependent variable (e.g., clotting time). ANOVA tests the null hypothesis that all group means are statistically equal. Before the analysis, the assumptions of residual normality and homogeneity of variances were verified using the Shapiro-Wilk test and Levene's test, respectively.

In cases where ANOVA yielded a statistically significant result ($p < 0.05$), Tukey's Honest Significant Difference (HSD) post hoc test was applied to identify specific differences between group means. This test controls the Type I error rate during multiple comparisons while maintaining good statistical power [18]. Results were visualized as bar charts, with letters indicating homogeneous groups according to Tukey's test. All statistical analyses were conducted using R software [19], with the agricolae package [20].

3. Results and discussion

3.1. Milk characteristics

The physicochemical composition of the reconstituted skim milk used as the Berridge substrate (12 %, w/v) was determined to characterize the experimental substrate. The results are summarized in Table 1.

Table 1. **Physicochemical characteristics of the milk used**
Таблица 1. **Физико-химические характеристики использованного молока**

Characteristic	Mean value
Total solids content (g·100 mL^{-1})	12.16 ± 0.04
Total protein (g·100 mL^{-1})	3.58 ± 0.12
Fat (g·100 mL^{-1})	0.11 ± 0.01
pH	6.8 ± 0.09
Ash (g·100 mL^{-1})	0.91 ± 0.14
Titratable acidity (g lactic acid · L^{-1})	1.8 ± 0.1

[1] ISO 6869:2000: Animal feeding stuffs — Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc — Method using atomic absorption spectrometry. Technical Committee : ISO/TC 34/SC 10.

[2] ISO 6731:2010: Milk, cream and evaporated milk — Determination of total solids content. Technical Committee : ISO/TC 34/SC 5.

[3] ISO 8968–1: 2014: Milk and milk products — Determination of nitrogen content — Part 1: Kjeldahl principle and crude protein calculation. Technical Committee: ISO/TC 34/SC 5.

[4] ISO 19662:2018 Determination of fat content — Acido-butyrometric (Gerber method). Technical Committee: ISO/TC 34/SC 5.

[5] ISO 26462:2010: Milk — Determination of lactose content — Enzymatic method using difference in pH. Technical Committee : ISO/TC 34/SC 5.

According to [21], the pH of milk varies between 6.60 and 6.91; values outside this range are indicative of abnormal milk. In the case of infection, the pH tends towards alkalinity or acidity, so the milk used can be considered normal. Given its satisfactory protein content (3.58 %) and low total fat content, this milk was considered a suitable substrate for determining the milk-clotting activity and clotting time of the enzyme studied. Optimal milk-clotting activity has been reported in milk with a total fat content below 1 % [3].

3.2. Crude extract characteristics

The characteristics of the chicken pepsin extract (protein content, milk-clotting activity, clotting strength, specific activity, and proteolytic activity) are presented in Table 2.

Table 2. **The main characteristic of the enzymatic extract**
Таблица2. Основные **характеристики ферментативного экстракта**

Characteristic	Chicken pepsin extract
Clotting strength (SU)	12957 ± 399
Milk-clotting activity (RU ml^{-1})	63.48 ± 0.21
Protein (mg mL^{-1})	43.49 ± 0.75
Specific activity (RU mg^{-1}) *	1.56 ± 0.19
Proteolytic activity (μg/mL · h)	0.91 ± 0.14
pH	7.9 ± 0.01
Color	Yellowish solution

* Specific activity (RU mg^{-1}) = clotting activity (RU mL^{-1})/protein content (mg mL^{-1}).

The clarified pepsin extract appeared as a yellowish solution. The protein content of the extract obtained was 43.49 ± 0.75 mg/mL, which is higher than the value reported by [3] (20.00 ± 0.00 mg/mL) and comparable to that reported by [6] (38.40 ± 0.40 mg/mL).

The result obtained indicates a proteolytic activity of 0.91 ± 0.14 μg/mL.h for chicken pepsin. This relatively high proteolytic activity of pepsin has been reported by several authors [22–24]. The milk-clotting activity was 63.48 ± 0.21 RU/mL, which is considerably higher than that reported by [3] (1.98 ± 0.02 RU/mL). The clotting strength was 12957 ± 399 SU. These differences may be attributed to factors such as age and feed of chickens, the maceration solution used, and extraction conditions.

3.3. Effect of enzyme volume on clotting time

One-way ANOVA revealed significant differences in clotting time across enzyme volume levels ($F = 9.484$, $p < 0.001$). Figure 1 illustrates the effect of enzyme volume on clotting time (in seconds).

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

Clotting time decreased significantly as the enzyme volume increased from 1 mL to 3 mL, reaching a minimum at 3 mL (group "c"). Beyond this point, clotting time increased, with the values at 5 mL and 7 mL belonging to groups "ab" and "a", respectively, and showing no significant difference from the extreme enzyme volume. Statistically, both 1 mL and 7 mL enzyme volume resulted in significantly longer clotting times than the 3 mL. These results suggest a non-linear relationship between enzyme volume and clotting time, with an optimal enzyme volume of 3 mL. A positive correlation (r>0.75) between the rate of gel formation, gel firmness, clotting time, and enzyme volume has been reported [3].

3.4. Effect of pH on clotting time

Figure 2 displays the relationship between pH and clotting time. The results show a strong decreasing trend in clotting time with increasing pH. This trend was confirmed by one-way ANOVA, which revealed a highly significant effect of pH on clotting time ($F = 51.19$, $p < 0.001$). Specifically, pH 5.6 and 5.8 were associated with the longest clotting times (group "a"), whereas pH 6.6 and 6.8 resulted in significantly shorter clotting times (groups "d" and "cd"). These findings suggest that milk-clotting activity of chicken pepsin increases with pH, with optimal values observed at pH 6.6–6.8.

It should be noted that the optimal pH for overall milk-clotting activity observed in the present study (6.6–6.8) is distinct from the pH optimum reported in the literature for the specific enzymatic hydrolysis of the Phe^{105}-Met^{106} bond of κ-casein (pH 5.1–5.3). This apparent discrepancy reflects the fact that milk clotting is a two-stage process: an enzymatic phase involving proteolytic cleavage of κ-casein, followed by a non-enzymatic aggregation phase governed by the colloidal properties of destabilized casein micelles [25]. While acidic conditions (pH ~5.1–5.3) maximize the rate of κ-casein hydrolysis by optimizing the catalytic geometry of the aspartic protease active site, the subsequent aggregation of para-κ-casein-bearing micelles is thermodynamically and kinetically more favorable at near-neutral pH values, where electrostatic repulsion is partially maintained but hydrophobic interactions can still drive network formation [26]. Consequently, the pH at which the integrated clotting activity — encompassing both proteolysis and aggregation — is maximized differs from the pH optimum of the proteolytic step alone. This two-stage pH dependence is consistent with observations reported for other plant- and animal-derived aspartic proteases used as alternative coagulants [27].

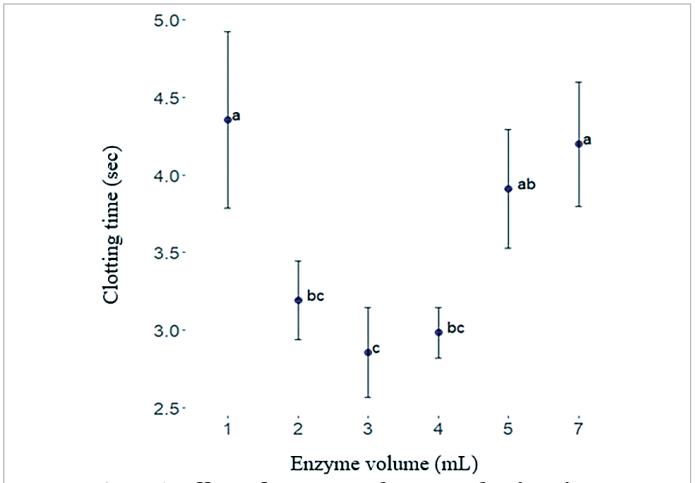

Figure 1. **Effect of enzyme volume on clotting time**
Рисунок 1. **Влияние объёма фермента на время свёртывания**

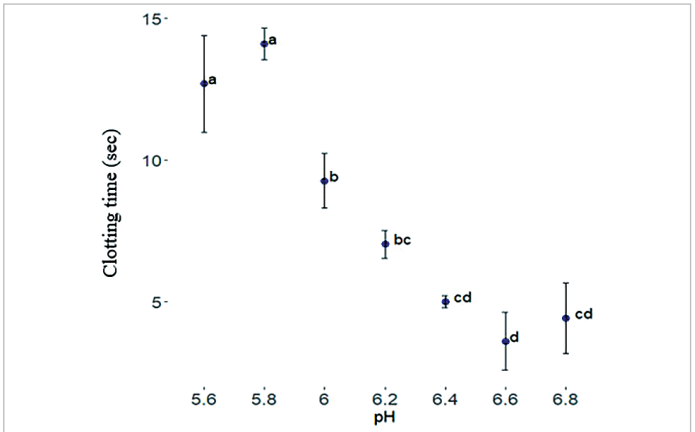

Figure 2. **Effect of pH on clotting time**
Рисунок 2. **Влияние pH на время свёртывания**

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

3.5. Effect of Temperature on clotting time

Figure 3 demonstrates that clotting time varies significantly with temperature ($F = 3.858$, $p = 0.0175$, one-way ANOVA). The longest clotting time was recorded at 40 °C, indicating slower clot formation.

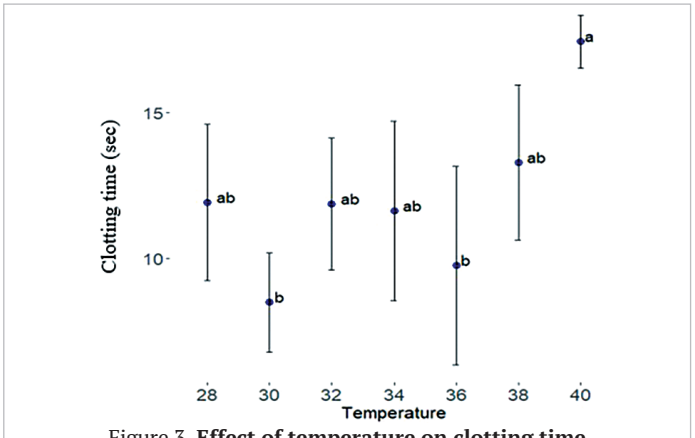

Figure 3. **Effect of temperature on clotting time**
Рисунок 3. **Влияние температуры на время свёртывания**

In comparison, 30 °C and 36 °C were associated with the shortest clotting times. Intermediate temperatures (28 °C, 32 °C, 34 °C, and 38 °C, group "ab") showed no significant difference from either group "a" or group "b". From 36 °C onward, clotting time showed an increasing trend, reaching its maximum at 40 °C.

The enzymatic clotting of milk is highly temperature-dependent. The rate of coagulum (gel) formation increases progressively as the temperature rises from 32 °C to 38 °C, which is near the optimum range for most milk-clotting enzymes. Below approximately 28 °C, gelation does not occur because the secondary (aggregation) phase is strongly inhibited, even if some enzymatic hydrolysis takes place. Increasing the clotting temperature primarily accelerates the secondary phase (micelle aggregation) by enhancing hydrophobic interactions and the mobility of rennet-altered micelles. However, at temperatures above ~38–40 °C, the overall clotting process slows down due to partial thermal inactivation or reduced stability of the coagulant enzyme itself [27].

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

3.6. Effect of $CaCl_2$ on clotting time

Figure 4 shows the variation in clotting time as a function of $CaCl_2$ concentration. Although Ca^{2+} addition is generally reported to shorten clotting time in rennet- or pepsin-induced milk clotting, no significant effect was observed in the present study ($F = 1.291$, $p = 0.323$). This lack of response suggests that, under our experimental conditions, calcium was not the limiting factor controlling flocculation kinetics. One possible explanation is that the native calcium content of the milk already provided sufficient ionic strength to support micelle aggregation, so that additional Ca^{2+} did not further enhance bridging or reduce electrostatic repulsion [28].

In addition, the enzyme-driven hydrolysis of κ-casein may have been the rate-limiting step, masking any potential effect of calcium on the secondary aggregation phase. Similar weak or non-significant responses to added $CaCl_2$ have been reported [26] in systems where micelle destabilization is primarily governed by enzymatic activity rather than ionic calcium availability or where calcium equilibrium between soluble and micellar phases is already near saturation. Therefore, while Ca^{2+} is theoretically expected to enhance aggregation through charge shielding and calcium bridging between casein micelles, its effect appears to be highly dependent on milk composition, enzyme type, and baseline mineral balance, which likely explains the absence of a significant effect in the present work [29].

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

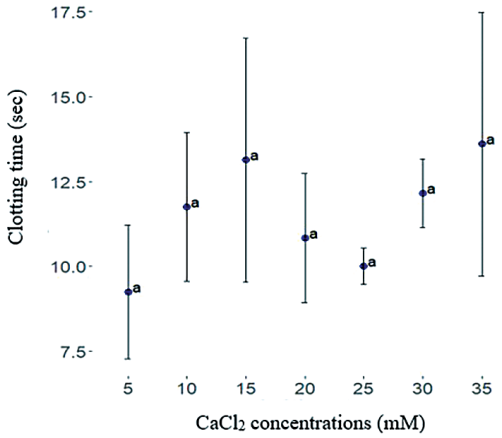

Figure 4. **Effect of $CaCl_2$ on clotting time**
Рисунок 4. **Влияние $CaCl_2$ на время свёртывания**

3.7. Effect of enzyme volume on flocculation time

Figure 5 illustrates the variation in flocculation time as a function of enzyme volume. At lower enzyme volumes of 1 and 2 mL, flocculation time was significantly longer, nearly 100 seconds. From 3 mL onwards, there was a marked reduction in flocculation time, with all higher volumes (3–7 mL) forming a single group ("b"), indicating no significant differences among them. These results suggest that increasing the enzyme volume beyond 2 mL leads to a substantial reduction in flocculation time, with no further significant reduction observed at higher volumes. As with any enzymatic reaction, the enzyme volume has a significant influence on the milk flocculation process. The flocculation time decreases with increasing enzyme volume [30–33].

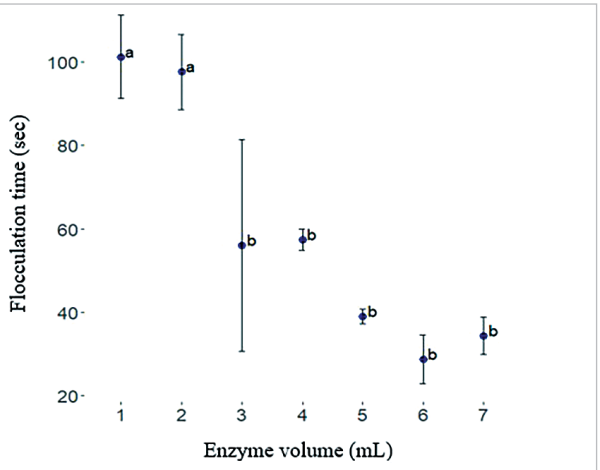

Figure 5. **Effect of enzyme volume on flocculation time**
Рисунок 5. **Влияние объёма фермента на время флокуляции**

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

3.8. Effect of pH on flocculation time

Figure 6 presents the variation in flocculation time as a function of pH. One-way ANOVA ($F = 49.15$, $p < 0.001$) followed by Tukey's HSD test revealed significant differences across pH levels, grouping the values into four homogeneous categories. Flocculation time was shortest at acidic pH values (5.6 and 5.8), both forming group "d". As pH increased, flocculation time gradually rose, reaching its maximum at pH 6.8, which belonged to group "a". Intermediate values (pH 6.0 to 6.6) formed groups ("cd", "c", and "b"), reflecting a clear upward trend. The observed pH dependence of flocculation time can be explained by the combined effects of casein micelle surface charge and the efficiency of enzymatic hydrolysis under different acidity conditions. At pH values closer to the isoelectric point of caseins, electrostatic repulsion between micelles is reduced, which facilitates aggregation and leads to a shorter flocculation time [34].

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

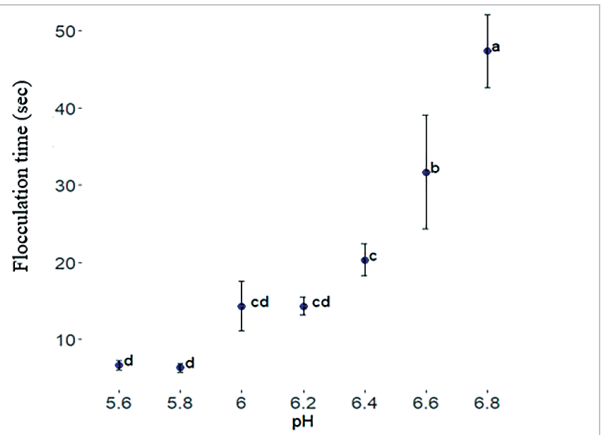

Figure 6. **Effect of pH on flocculation time**
Рисунок 6. **Влияние pH на время флокуляции**

3.9. Effect of temperature on flocculation time

Figure 7 illustrates the relationship between flocculation time and temperature. One-way ANOVA revealed a statistically significant effect of temperature on flocculation time ($F = 4.889$, $p = 0.0068$). According to Tukey's HSD post hoc test, the data were grouped into partially overlapping homogeneous groups. The shortest flocculation time was recorded at 36 °C (group "b"), indicating optimal performance at this temperature. In contrast, longer flocculation times were observed at both lower (28–30 °C) and higher (40 °C) temperatures. This suggests a U-shaped relationship, with moderate temperatures prompting faster flocculation than temperature extremes.

The influence of temperature can be attributed to its impact on two main processes: the enzymatic reaction itself and the subsequent clotting

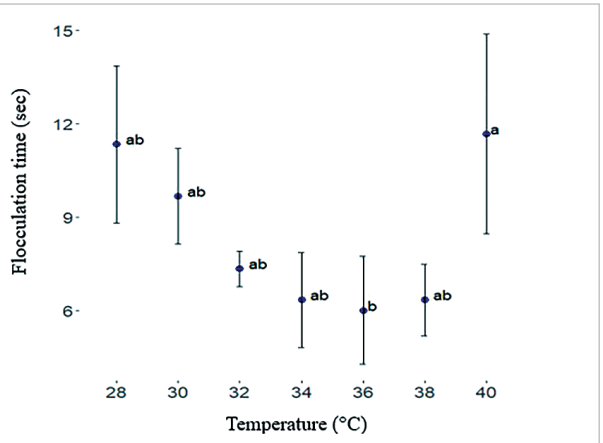

Figure 7. **Effect of temperature on flocculation time**
Рисунок 7. **Влияние температуры на время флокуляции**

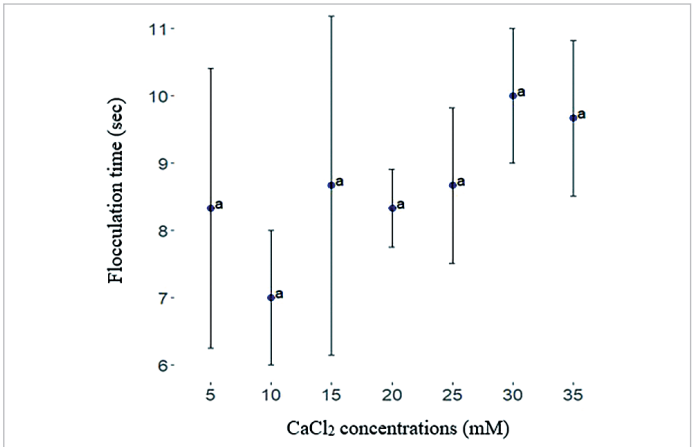

Figure 8. **Effect of $CaCl_2$ concentration on flocculation time**
Рисунок 8. **Влияние концентрации $CaCl_2$ на время флокуляции**

phase, particularly the aggregation stage. Milk clotting typically occurs at temperatures of 18 °C or above, as hydrophobic interactions become the dominant force driving the aggregation of hydrolyzed micelles [34–38]. Moreover, enzyme thermal inactivation is associated with partial unfolding of its native three-dimensional structure due to the disruption of non-covalent interactions, including hydrogen bonds and hydrophobic interactions.

Under more severe heating conditions, these structural alterations may become irreversible, involving not only conformational changes but also covalent modifications of the primary structure, including chemical damage and irreversible alterations of amino acid side chains such as oxidation, deamidation, or peptide bond cleavage. In the present study, the increase in flocculation time observed at 40 °C is therefore consistent with the onset of thermal inactivation of chicken pepsin, whereby progressive structural alterations at this temperature reduce the enzyme's proteolytic efficiency toward κ-casein, slowing the rate of Phe^{105}-Met^{106} bond hydrolysis and consequently delaying the destabilization of casein micelles and the onset of the secondary aggregation phase [39].

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

3.10. Effect of $CaCl_2$ on flocculation time

Figure 8 illustrates the variation in flocculation time as a function of $CaCl_2$ concentration. One-way ANOVA revealed no significant effect of $CaCl_2$ concentration on flocculation time ($F = 1.291$, $p = 0.323$).

The addition of calcium chloride ($CaCl_2$) to milk is generally reported to reduce pH slightly and enhance protein aggregation, thereby accelerating clotting [40]. In agreement with previous studies, the effect of calcium is known to be strongly pH-dependent, with improved gel formation typically observed at near-neutral pH values (≈6.5–6.7) [41]. Under such conditions, Ca^{2+} ions promote micellar aggregation by reducing electrostatic repulsion and facilitating calcium bridging between casein micelles [42,43].

However, in the present study, no statistically significant effect of $CaCl_2$ concentration on flocculation time was observed. This apparent discrepancy may be explained by several factors. The absence of a significant $CaCl_2$ effect on flocculation time in the present study may be attributed to three concurring factors: the baseline calcium content of the reconstituted Berridge substrate may have already reached the threshold for near-optimal micelle aggregation [44]. the proteolytic activity of chicken pepsin may have been the dominant rate-limiting factor under the present experimental conditions, thereby subordinating the contribution of calcium to the overall clotting process [45].

Each point represents the mean clotting time; error bars represent standard deviation (SD). The letters indicate homogeneous groups according to Tukey's test at the 5 % significance level.

4. Conclusion

These findings confirm that chicken pepsin possesses effective milk-clotting activity under optimized conditions and represents a promising, and biologically sustainable alternative to conventional rennet. However, the present study was conducted under controlled conditions using a reconstituted skim milk substrate, and the transferability of these findings to actual cheesemaking conditions remains to be established. Future work should therefore focus on direct cheesemaking trials evaluating the impact of chicken pepsin on yield, texture, proteolysis profiles, and sensory properties, in order to fully assess its practical potential as a rennet substitute in dairy processing.

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Contribution	Критерии авторства
Authors equally relevant to the writing of the manuscript, and equally responsible for plagiarism.	Авторы в равных долях имеют отношение к написанию рукописи и одинаково несут ответственность за плагиат.

Conflict of interest	Конфликт интересов
The authors declare that there is no conflict of interest.	Авторы заявляют об отсутствии конфликта интересов.