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Original Article

Use of *Esienia fetida* Worms to Produce Peptone for *Clostridium perfringens* Vaccine Production

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Abstract

Concurrent with an increase in the human population on the earth, more than ever, the creation of energy and maintenance of health is necessary, and nowadays, various sources of energy supply are being developed. The general global view in this regard is to provide protein and energy from available and cheap sources. Iran is no exception to this general rule, only in the field of ensuring the health of livestock resources every year, about 10 tons of peptone is needed for producing clostridial vaccines. Vermicomposting worms (*Esienia fetida*) with high protein percentages and rapid reproductions are a suitable source for peptone production. Based on this, the vaccine strain of *Clostridium perfringens* type D cultivated in two different media contain peptone produced from worms and meat peptone. The growth rate, epsilon toxin (ETX), and alpha toxin (CPA) of *Cl. perfringens* have been compared in two media. The results showed that the growth rate of bacteria in the worm peptone medium in 48 h was 22% higher than that of the meat peptone. Additionally, the activity of alpha toxin (phospholipase C) was in worm peptone 15% higher than meat peptone during 80 min of measurement. Regarding epsilon toxin lethality, all three mice of the N-worm peptone group died, while all three mice of the meat peptone group survived even 72 h after injection. The average survival time of mice in the N-worm peptone group was 1700 min. Therefore, we suggest the worms' protein is more suitable than industrial meat in peptone production for vicinal propose. To eliminate the need for hydrolyzed protein in the production of vaccines in the future, we suggest an increase in the fields of employment and the development of fertilizer and worm farms in Iran.

Keywords: *Clostridium perfringens*, Epsilon toxin, *Esienia fetida* worm, Peptone, Phospholipase C

1. Introduction

1.1. Needs to Prepare Hydrolyzed Protein

Developing countries often have serious problems with food supply in terms of accessing and consuming proteins that have higher nutritional value than other nutrients. Unfortunately, the diet of millions of people in countries is more dependent on using carbohydrates to provide energy for their bodies. However, the carbohydrate diet often lacks amino acids. Therefore, it is limited by not having essential amino acids. Accordingly, efforts have been made to obtain protein from unconventional sources that can be used in various food products for human consumption (1). The most basic application of hydrolyzed protein in biotechnology is providing a source of nitrogen for bacterial culture environments on an industrial and specialized scale. It is also a source of nitrogen for the cultivation of microbial, plant, animal, and insect cells on a laboratory and industrial scale (2).

1.2. History of Protein Hydrolysis

The first acid hydrolysis of protein for amino acid analysis was reported in the early 1950s (3). Although before that in 1820, the first acid hydrolysis of protein (peptone production) had been carried out. However, it took several decades to commercialize this process, and it is still ongoing (2, 4). In the field of protein hydrolysis from different sources, many studies have been done, including protein analysis of vermicomposting worms (*Eisenia fetida*) to supply human and animal food (1). *Eisenia fetida* is classified in phylum: *Annelida*, class: *Oligochaeta*, order: *Haplotaxia*, and the family: *Lumbricidae* (5). In addition, the flour prepared from vermicomposting worms has been used as an economic source for the production of lactic acid in fermentation conditions due to their high amount of nitrogen and protein (6). In Iran, intestinal wastes and viscera of rainbow salmon and chicken have been hydrolyzed with alkalase (7).

1.3. Relative Evaluation of the Need for Protein Sources at Razi Institute

It is necessary to acquire the technical knowledge of producing peptone from different sources for vaccine production in Iran. The farms producing vermicomposting using *Eisenia fetida* worms are increasingly developing and expanding in Iran. The amount of protein in this organism is very high, its protein content is 61.85%, with 11.13% fat, and 7.8% ash in one gram of dry weight (1). This research was carried out with the priority to obtain the technical knowledge of vaccine production from hydrolyzed protein from this cheap and accessible source.

1.4. Peptone Production

Peptones, as a main ingredient of media, provide carbon, nitrogen, minerals, and growth factors needed to support the metabolic requirements of microbial and mammalian cells. Therefore, they cause cell proliferation and production. Protein sources that are commonly used as raw material in peptone production include meat, gelatin, soy, casein, and whey (milk proteins) (Persian article) (8).

1.5. *Clostridium perfringens* Bacterium

Clostridium perfringens was first identified during the autopsies of patients who had cancer or tuberculosis 8 h postmortem at the end of the 19th century. *Cl. perfringens* is an anaerobic and spore-forming gram-positive bacterium (9, 10). The bacterium widely spreads in the environment, especially in the soil, water, sewage, and gastrointestinal tracts of healthy humans and animals (11). It is the cause of histotoxic and enterotoxin diseases in humans and domestic and wild animals. The bacterium is the cause of gas gangrene and mild enterotoxaemia in humans (12). *Cl. Perfringens* can produce more than 20 toxins. Not all of these toxins are produced by all strains (12) *Cl. perfringens* categorizes in five types A to E based on four major toxin production CPA, beta, ETX, or iota (13). *Cl. perfringens* type D has two specific major toxins, CPA, and ETX (11).

1.5.1. Epsilon Toxin (ETX)

Epsilon toxin is the third strongest toxin among bacterial toxins and can be dispersed as an aerosol and is known as a bioterrorism agent (9, 14). Epsilon toxin is produced by types D and B of *Cl. perfringens*. This toxin is the cause of enterotoxaemia in animals, such as goats and sheep, and less often cattle (15). Although ETX is initially inactive as a protoxin and becomes an active toxin under the effect of digestion with trypsin enzyme. In addition, ETX prototoxin in the culture medium is probably activated under the effect of bacterial proteolytic enzymes such as kappa and lambda (16).

1.5.2 Alpha Toxin (CPA)

Alpha toxin is the first bacterial toxin known as an enzyme (16). This enzyme is a zinc-containing phospholipase C, and contains two structure domains whose molecular weight is 43 kDa (11). Alpha toxin catalyzes the hydrolysis of lectin molecules into phosphoryl choline and water-insoluble 1, 2-diacylglyceride. Alpha toxin is used to study the biological and lipid structure of the membrane and the relationship between this structure and the membrane function (16).

Alpha toxin is produced by all types of *Cl. perfringens* (11). It is also a pathogenic factor in several human and domestic animal diseases and performs its function by activating the release of arachidonic acid (17). In addition, CPA has lethal, necrotizing, hemolytic, and cytolytic activities (16, 18, 19). Alpha toxin is the main cause of gas gangrene, and its action is weakened by antibodies against the CPA toxoid vaccine. Whatever the CPA used for the vaccine production is purer the vaccine or toxoid is more effective (17). In the current research, the growth and toxin production of the *Cl. perfringens* (type D) vaccine strain was evaluated in hydrolyzed protein media prepared from *Esienia fetida* worms and in the meat peptone media. On the other hand, the amount of toxin secreted by the bacterium in both media, as well as the growth were measured.

2. Materials and Methods

2.1. Production of Peptone from *Eisenia fetida* Worms

The *Eisenia fetida* worms were purchased from the worm breeding farm around Mashhad, (Khorasan province, Iran) and were transferred alive in a natural substrate containing cow dung to the Research Department of Razi Institute, Mashhad branch. The worms were transferred to a container of water containing the same size of clean sand to empty the materials of the digestive system for 24 h. After this period, the worms were washed with water several times, and weighed with a Sartorius laboratory scale in wet conditions. The production of peptone from worms was done based on a method by Noruzy Mogadam and Banaei (8). Briefly, steps of washing, weighting, homogenization, fat removal, steam hydrolysis, and drying were performed. The details of the steps are shown in table 1. Finally, 10 gr N-worm peptone produced after two days work on 40 gr worms.

2.2. Preparation of Culture Media from Worm and Meat Peptone

The culture medium preparation for *Cl. perfringens* type D was carried out based on a method proposed by Ardehali and Darakhshan (20). For this purpose, two culture mediums were prepared in two Erlenmeyer flasks with a volume of 50 ml from two different peptones: meat peptone (HiMedia Laboratories, LLC) and N-worm peptone. The conditions and formula for preparing both cultures were the same. Therefore, except peptone, all trace elements and vitamins were used at the same amount in each of the two media. Both media were autoclaved and sterilized for 15 min at 121℃.

2.3. Bacterial strains and culture conditions

The vaccine strain of *Cl. perfringens* type D from liver broth medium (working seed) was used for cultivation. For this purpose, one percent of *Cl. perfringens* type D from liver broth was cultured in meat and worm peptone mediums. Then, the cultivated Erlenmeyer flasks were placed in an aerobic jar, and absolute anaerobic conditions were provided to them with Anoxomat (Mart, the Netherlands). Next, the anaerobic jar was incubated at 37℃ for 72 h.

Table 1. Steps and conditions of peptone production from Eisenia fetida worms in Noruzy Mogadam and Banaei (8) method

no	Treatment		Time required (Min.)	Temperature (ºC)
1	Cleaning the digestive system of worms in the water		1440	25
2	Washing with water		60	25
3	Homogenization		30	25
4	Fat removal	Initial reaction in chloroform	30	25
		Secondary reaction in chloroform	30	60
		Centrifuge(3000 rpm)	30	25
		Evaporation of chloroform	300	70
5	Steam hydrolysis		600	110
6	Drying		180	70
7	Total Time		2700	-

2.4. Monitoring and Determination of the Growth Phase

The growth curve of bacteria was measured based on the Duracova, Klimentova (9) method with a slight change. For this purpose, the optical density of the cultures' growing bacteria was measured at 600 nm wavelength with a spectrophotometer (Shimadzu, Japan) at intervals of 24 h. In addition, according to the Si method (21), the CFU/ml has been calculated and reported. As the method, the relationship between CFU/ml on OD=600 for *Cl. perfringens* concludes from CFU/ml= $(200\times OD_{600}/6)\times 10^6$

2.4.1. Toxic Activity

To measure the toxic activity based on the method proposed by Fernandez-Miyakawa, Marcellino (22), the toxin was first prepared by centrifugation (Sigma-3-18K) and filtration; then, the surviving time and the activity of phospholipase C were evaluated. In this method, a survival time test is used to measure ETX, although, in this test, ETX is not subjected to enzymatic digestion to become a complete toxin. To perform the test, two groups of three mice were injected with the filtered supernatant solution of the medium. Moreover, measuring the activity of phospholipase C is the main criterion for the CPA assessment.

3. Results

The results of the *Cl. perfringens* growth curve in the media are shown in figure 1. The maximum growth occurred within 48 h in both mediums. Although after this period, the growth curve in 72 h compared to 48 h showed a slight decrease in both media. The growth rate of bacteria in the N- worm peptone during 24, 48, and 72 h was higher than the meat peptone.

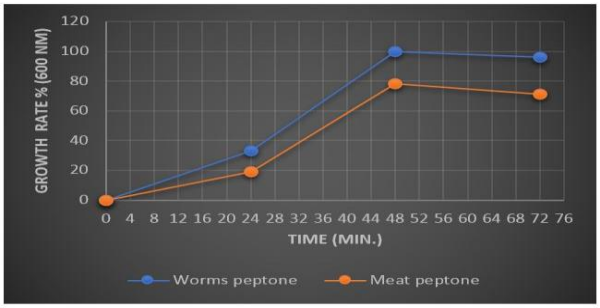

Figure 1. Growth rate of *Cl. perfringens* in two media contain: N-worm peptone, Himedia meat peptone

The results of calculating the CFU/ml of culture medium suspension in 24, 48, and 72 h are shown in figure 2. These qualitative results are suitable for comparing the number of bacteria grown in both media. The number of bacteria in the N-worm peptone medium was 32, 50, and 58 ($\times 10^6$) units/ml more than the meat peptone medium, and the growth rate of the bacterium in the N-worm peptone medium was 1.7, 1.3, and 1.4 more than in meat peptone medium in 24, 48 and 72 h, respectively.

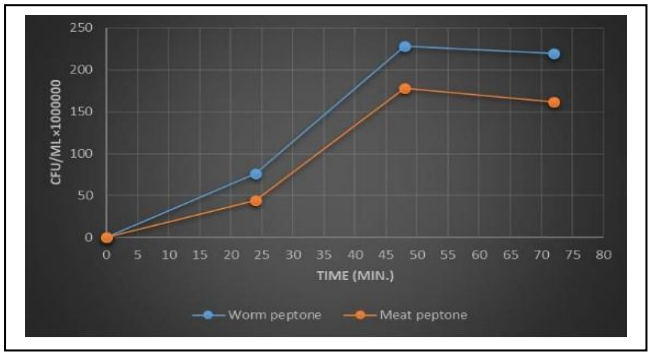

Figure 2. The number of *Cl. Perfringens* (CFU/ml) in two media: N-worm peptone, Himedia meat peptone

The results of the ETX effect and survival time of *Cl. perfringens* type D injection in mice from N-worm and meat peptone media are shown in table 2. The results of the lethality of *Cl. perfringens* are usually attributed to the ETX without considering the other toxins' effect due to its high toxicity and lethality. All three mice of the N-worm peptone group died, while all three mice of the meat peptone group survived even 72 h after injection. The average survival time of mice in the N-worm peptone group was 1700 min.

The results of phospholipase C activity are shown in figure 3. These results are related to measuring the effect of CPA. The activity of the CPA of *Cl. perfringens* in both media was the same within 10 min. The CPA activity in both groups reached its max point at 20 min. Gradually, the enzyme activity decreased during the 20th min to the 100th min in both groups. During this period, the activity of CPA toxin from the N-worm peptone group was higher than the meat peptone group. Alpha toxin (phospholipase C) activity of *Cl. perfringens* grown in N-worm peptone was about 15% higher than the meat peptone during 80 min.

Table 2. The epsilon toxin effect and survival time of *Cl. perfringens* type D injection in mice from N-worm and meat peptone media

Treatments	Number of mice injected	Number of survival mice after 48 (hr)	Number of death mice after 48 (hr)	Average of survival time (min)
Worm peptone	3	0	3	1700
Meat peptone	3	3	0	All alive

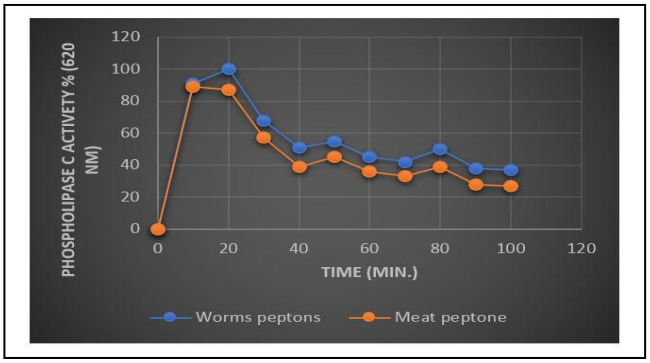

Figure 3. The alpha toxin activity (phospholipase C) of *Cl. perfringens* in two media: N-worm peptone and Himedia meat peptone

4. Discussion

It is clear that the growth factors in the medium that result in the bacterial growth is different from factors that initiate exotoxin secretion. The results of the comparison between soybean peptone medium with meat media in Razi Institute revealed that the bacteria growth was the same in both meat and soy protein sources. Nevertheless, in examining the toxin of different types of *Cl. perfringens*, the amount of exotoxin caused by cultivation in soybean medium was less than meat peptone. This reduction in toxin production may be caused by the ratio of essential amino acids that is not suitable for the stimulation, production, and secretion of exotoxin in soy media (23).

It should be borne in mind that this is the first time this scale of research has been carried out using worms' peptone, and comparable results have not been reported yet.

However, in comparison, the amount of protein in soy is less than in worms. Consequently, the amount of protein in worms is 61.85% in one gram of dry weight (1), while dry soybeans have 37% protein (24). In addition, soybean meal after oil extraction has about 50% protein (24, 25). Therefore, according to the amount of protein, choosing worms as a source of protein is preferable to soy. Separating oil and carbohydrate from plant sources such as soy to produce peptone requires spending a lot of capital and time.

Using the reported Si method (21) formula to calculate the CFU/ml is suitable, although it may not be accurate; its results are valuable for comparison.

Although ETX of *Cl.perfingens* is the third strongest toxin among bacterial toxins, it secrets as a prototoxin, and its greatest effect is after enzymatic hydrolysis, especially by trypsin. In the study, the lethality effect of ETX prototoxin in the mice (without per-enzymatic hydrolysis) may be related to proteolytic cleavage in the cell surface by furin.

In the field of measuring CPA, which is a phospholipase C, it is clear that the higher the amount of phospholipase C produced in the culture medium, the more effective it will be on the egg lectin substrate, and as a result, there will be more product, which indicates more activity.

The reason for the higher bacteria growth in the worm peptone medium compared to imported meat

peptone is probably due to several reasons. Firstly, the peptone produced from worms has a very high purity and does not contain accompanying substances, while the peptone produced on an industrial scale often does not have 100% purity. Secondly, the peptone produced from the worm may be richer than the comparable meat peptone due to having more essential amino acids. Thirdly, the peptone produced from the worm was used fresh, while the peptone from the meat was not fresh.

It should be noted that this research was conducted at the preliminary and laboratory levels, and these results should be confirmed at the semi-industrial and industrial levels.

Authors' Contribution

Study concept and design: H. N. M.
Acquisition of data: H. N. M. and B. M.
Analysis and interpretation of data: H. N. M., M. H. and H. R. F.
Drafting of the manuscript: H. N. M., M. H. and H. R. F.
Statistical analysis: H. N. M., M. H. and M. M. J.
Administrative, technical, and material support H. N. M. and H. J.

Ethics

All the ethical standards were approved by the ethics committee of the Razi Vaccine and Serum Research Institute Mashhad Branch, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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