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EXTRACTION AND PRECIPITATION OF PEPSIN FROM BOVINE GASTRIC TISSUES

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Optimum conditions for the extraction and precipitation of bovine pepsin from young buffalo were studied. Changes in pH, temperature and extraction time significantly affected the extraction of pepsin. Maximum pepsin activity (222 unitsg^{-1}) was observed when minced gastric tissues were suspended in acidic solution of pH 2.2 at 40°C for 18 hr. Pepsin was precipitated from the extract by adding sodium chloride at the rate of 200g^{-1}. Pepsin activity in camel, cow, buffalo and chicken gastric tissues, under these conditions was found to be 101, 141, 190 and 222 units respectively, while 172 unitsg^{-1} pepsin activity was observed in cases of sheep and goat. These gastric tissue samples of different animals were kept at -10°C for six months and a gradual decrease in pepsin activity by 18-68% was observed during storage.

Key words: Pepsin, Bovine, Extraction.

Introduction

Pepsin is a proteolytic enzyme which is present in variable amounts in the gastric tissues of all kinds of vertebrates [1-5]. It has been observed that the amount of pepsin in cattle, sheep, swine and rabbit is 1300, 1200, 1490 and 1175 $mg^{-1}g$ respectively [6]. It has also been reported that younger animals have relatively more pepsin in gastric tissues than older ones [7]. Pepsin has many useful industrial applications. It is used for making cheese and protein hydrolyzates [8-10]. It is also used as digestive enzyme in pharmaceutical preparations.

There are various methods for the extraction of pepsin from the gastric tissues of the animal but the literature regarding optimum extraction conditions is scanty. Pepsin is usually extracted by suspending minced gastric tissues in acidic solutions containing hydrochloric acid, sulphuric acid or phosphoric acid. Valles and Furet [11] obtained pepsin from bovine abomaseum with acid extraction technique using 0.2M HCl and precipitated the enzyme by the addition of ethanol at pH 1.0. Herriott and Northrop [12] isolated pepsin in crystalline form from the extract of swine fundus mucosa with the saturated solution of ammonium sulphate as precipitating agent. Kostka *et al.* [13] prepared pepsin from chicken and duck while Vladimir *et al.* [14] attempted to extract pepsin from yak and camel gastric tissues using acidic solution of sulphuric acid. Kageyama and Takahashi [15] extracted it from gastric mucosa of Japanese monkey with 0.02 M sodium phosphate buffer and precipitated the enzyme with the addition of soil ammonium sulphate. Besides these, many other methods for the extraction of pepsin are available in the literature which are lengthy, complicated and laborious. Moreover, these methods result in very poor yield of pepsin. Therefore, there is a need to develop a simple and efficient method of pepsin extraction from gastric tissues of various animals.

Present work was undertaken to optimize the conditions (pH, temperature and time period of extraction) for the development of a simple and efficient pepsin extraction procedure from bovine gastric tissues. Since the temperature in summer goes too high, so gastric tissues are kept in deep freezer to avoid deterioration. Hence, the effect of cold storage (-10°C) on the activity of pepsin was also studied.

Materials and Methods

Gastric tissues of young buffalo (approx.2 years old) and other animals were collected from the local slaughter house soon after slaughtering the animals. The contents of stomach were removed and washed with chilled water immediately. The washed tissues were put in ice box and brought to laboratory for processing. The offals and fatty layers were removed before mincing. Minced tissues were packed in plastic bags and preserved in the freezer for later use.

Extraction of pepsin. Minced gastric tissue was mixed well with water of different pH containing 1.0% boric acid keeping substrate to water ratios 1: 1.00, 1: 1.25, 1: 1.50, 1: 1.75(w v^{-1}). The desired pH of the mixture was adjusted with 0.1N hydrochloric acid on pH meter and then incubated at different temperatures for different time periods with frequent stirring. After incubation, the contents were filtered and pepsin activity was assayed in the filtrate. HCl solution, HCl buffer and citric acid solution of pH 2.2 were also used for extraction of pepsin. Composition of these solutions is given in Table 1.

Precipitation of pepsin. Sodium chloride and ammonium sulphate were used as precipitating agents in this study. Different amounts of the precipitating agents were added slowly

with continuous stirring into the filtrate and mixture was allowed to stand for about 20 minutes to complete the precipitation. Precipitate of crude pepsin was collected by filteration and then dried at ambient temperature.

Assay of pepsin. The method described by Anson [16] was used for the determination of pepsin activity in the enzyme extract obtained from gastric tissues. The enzyme reaction was started by the addition of 1 ml of 2.5% haemoglobin that had previously been acidified to pH 2.5 with HCl after incubation for 10 minutes at 37°C and the reaction was terminated by adding 10 ml of 5% trichloro acetic acid. The mixture was then filtered through Whatmann No.576 filter paper. The optical density of the filtrate was measured at 280 nm on a spectrophotometer.

Protein concentrate. The offals were minced, mixed with water and autoclaved for 30 minutes. Fat was skimmed off from the top, excess of water was removed by filtration through cheese cloth and the tissue was dried at 60 ± 5°C. Protein concentrate was obtained by mixing the autoclave dried offals with the residue left after extraction of pepsin from gastric tissue. Chemical analysis of protein concentrate was carried out according to A.O.A.C. method [17].

Microbiological examination. Pathogenic microorganisms such as *E.coli, Salmonella* sp. and *Clostridium* sp. were isolated and identified by the methods as described in the Handbook of Microbiology published by Merck [18].

TABLE 1. EFFECT OF DIFFERENT SOLUTIONS ON THE EXTRACTION OF PEPSIN*

Extractants** (pH 2.2)	Material Extractant	Temp. (°C)	Time (hr)	Units/g tissue weight
Citric Acid Solution	1: 1.25	40	18	119
HCl-Buffer	1: 1.25	40	16	140
HCl-solution	1: 1.25	40	18	205

*Average of three determinations; ** Composition of extractants. *Citric acid solution:* pH 2.2 was adjusted by addition of 5 g citric acid in 100 ml water; *HCl Buffer:* (pH 2.2 contain 25 ml 0.2 M KCl and 3.35 ml 0.2 M HCl in 100 ml water; *HCl solution:* pH 2.2 was adjusted by the addition of 8 ml of 0.1 N HCl in 100 ml water.

Results and Discussion

Optimum conditions for the extraction of pepsin. Extraction of pepsin from athe bove mentioned gastric tissues were significantly affected as a result of change in pH, temperature and time period of incubation.

pH of the solution played a significant role in the extraction of pepsin. It is apparent from the results that conversion of pepsinogen to pepsin varied appreciably at different incubation pH (1.2, 1.7, 2.2 and 2.7). Highest activity of pepsin was found to be at pH 2.2 Maximum activity of pepsin in the esxtract was 192 units g^{-1} of fresh tissue weight when minced gastric tissues were suspended in acidic solution of pH 2.2 at 40°C for 18 hr (Table 2). At pH 2.7, activity of pepsin showed marked decline at all temperatures and incubation periods.

Variable amounts of pepsin were obtained from the minced gastric tissues at temperatures ranging from 30 - 45°C (Table 2). Maximum activity of pepsin in the extract was 89 units g^{-1} at 40°C on mixing the tissues with acidic solution of pH 1.2 after 18 hrs. Similarly, maximum pepsin activity was also observed at 40°C when pH of the solution was raised from 1.2 to 2.7. However, significant reduction in pepsin activity was observed when temperature was raised from 40 to 45°C.

Experiments were also conducted to find out the effect of extraction time on the activity of enzyme. It is apparent from Table 2 that acidic solution of pH 1.2 extracted only 38 units of pepsin from one gram of fresh gastric tissues after 6 hr. The extraction of enzyme seemed to increase with the increase in the extraction period. The optimum time for the maximum enzyme extraction was 18 hr. Maximum pepsin activity was 70 units g^{-1} after 18 hr at 30°C at pH 1.2. No further increase in the amount of pepsin was observed after 18 hr incubation.

Effect of different material-extractant ratios on the activity of pepsin was also studied at 40°C for 18 hr. The activity of pepsin in the filtrate was maximum (212 units g^{-1}) when the material extractant ratio was 1: 1.25 (Table 3).

TABLE 2. EFFECT OF pH, TEMPERATURE AND TIME PERIOD ON EXTRACTION OF PEPSIN*.

Extraction time	Pepsin Activity (units/g fresh tissue weight)															
	pH-1.2				pH-1.7				pH-2.2				pH-2.7			
(hr)	30**	35	40	45	30	35	40	45	30	35	40	45	30	35	40	45
6	38	44	68	59	131	140	147	141	148	156	172	154	129	132	146	135
12	51	60	79	62	134	146	151	141	152	159	181	160	135	140	149	137
18	70	76	89	70	141	148	159	147	161	173	192	171	147	151	162	146
24	70	75	84	69	144	163	171	160	159	170	190	167	161	170	175	150
30	72	74	86	70	145	175	183	170	154	165	172	161	156	169	170	152

*Average of three determinations. **Extraction temperature °C.

TABLE 3. EFFECT OF MATERIAL EXTRACTION RATIO ON PEPSIN ACTIVITY AT pH 2.2.

Material Extractant Ratio	Temp. (°C)	Time (hr.)	Units/g tissue weight*
1: 1.00	40	18	170
1: 1.25	40	18	212
1: 1.50	40	18	155
1: 1.75	40	18	130

*Average of three determinations.

TABLE 4. EFFECT OF DIFFERENT SALTS ON THE PRECIPITATION OF PEPSIN FROM THE FILTERATE.

Precipitating Agent	Amount added (g/litre)	Crude pepsin Yield*(g/kg)	Pepsin Activity units/mg dry wt.
Sodium Chloride	50	8.20	949
	100	16.50	950
	200	18.70	971
Ammonium Sulphate	50	4.40	860
	100	8.91	865
	200	10.29	880

*Average of three determinations.

TABLE 5. EFFECT OF STORAGE ON PEPSIN ACTIVITY OF DIFFERENT ANIMAL'S GASTRIC TISSUE.

Animals	Units-1g tissue weight*						
	0**	1	2	3	4	5	6
Buffalo	190	185	181	167	89	76	71
Cow	141	133	116	85	72	69	59
Goat	172	166	165	161	150	139	112
Sheep	172	167	165	162	149	137	115
Camel	101	91	86	73	61	55	47
Chicken	222	210	205	202	198	190	181

*Average of three determinations. **Storage time in months.

A decline in pepsin activity by 38% was observed when the material-extractant ratio was increased to 1: 1.75.

On the basis of these findings, it may be concluded that maximum amount of pepsin can be extracted by mixing the minced gastric tissues with HCl solution of pH 2.2 in 1: 1.25 ratio at 40°C for 18 hr.

Extraction of pepsin using different solutions. It is evident from the results (Table 1) that maximum pepsin activity was 205 units g^{-1} with HCl solution at 40°C after 18 hr, while the minimum activity of pepsin was observed with citric acid solution. HCl buffer also extracted pepsin but the activity was comparatively less than the HCl solution. It indicates that presence of potassium ions in HCl buffer retarded the activity of pepsin to some extent. Therefore, HCl solution of pH 2.2 can be effectively used for the extraction of pepsin from gastric tissues of the animals.

Precipitation of pepsin. Crude pepsin was precipitated from the filtrate by the addition of variable amount of sodium chloride and ammonium sulphate. Sodium chloride was found to be significantly better precipitating agent as compared to ammonium sulphate. Maximum crude pepsin (18.7g) was obtained from one kg of fresh gastric tissues when sodium chloride at the rate of 200g^{-1} litre was added slowly with constant stirring in the filtrate (Table 4), while 10.29 gkg^{-1} crude pepsin was recovered with ammonium sulphate. The activity in crude pepsin (dried) material was found to be 971 units mg^{-1} which can be further increased on purification.

Effect of frozen storage on pepsin activity. The minced gastric tissue samples of different animals were stored at -10°C in a deep freezer for six months. The samples were taken out at interval of one month and analysed for pepsin activity. It is apparent from Table 5 that pepsin activity in fresh gastric tissues of different animals significantly varied (from 101 to 222 units g^{-1}) which decreased from 18 to 68% during six months storage at -10°C. These results are in agreement with the findings of other workers [19,20] who reported variable amounts of pepsin in different species of ruminants, while Djordjevic *et al.* [21] observed a decline in pepsin activity by 20% on freeze storage of hog stomach.

Fresh gastric tissues of buffalo contained 190 units of pepsin which decreased to 167 units g^{-1} after three months storage. However, pepsin degradation took place rapidly as the storage progressed and the sample contained only 71 units pepsin/g at the end of six months (Table 5). Similar trend was observed when cow gastric tissue samples were kept at-10°C for six months. Hence, it is obvious that under these conditions, buffalo and cow gastric tissues should not be stored for more than three months. Sheep and goat tissue contained 172 units pepsin g^{-1}. It also decreased but at a slower rate as compared to buffalo and cow gastric tissues. So it seems feasible to extract pepsin from sheep and goat tissues upto four months storage. These results are consistent with the findings of other workers [22] who also reported that sheep pepsin was more stable than cattle pepsin. Fresh gastric tissues of camel contained 101 unit pepsin g^{-1} which decreased to 47 units g^{-1} after six months. Among various animal gastric tissues examined, chicken gastric tissue was found to contain maximum pepsin activity (222 units g^{-1} and it decreased to 181 units g^{-1} after six months frozen storage. This means that chicken pepsin was more stable as compared to pepsin obtained from other sources studied during this study.

Utilization of protein concentrate. Protein concentrate was obtained as a by product after the extraction of pepsin from gastric tissues of the cattle. It contained 68.4% protein

and 5.39% minerals whereas 13.8% fat was also present in this by-product. Microbiological examination of this product showed that it was free from pathogenic microorganisms (*E. coli, Salmonells* sp. and *Clostridium* sp.) and bacterial counts were found to be 7000 per gram. These results indicate that this could be utilized as a protein ingredient in poultry feed.

Conclusion

Optimum conditions for the extraction and precipitation of bovine pepsin from young buffalo were standardized. The following standardized procedure was used: 100 grams of minced fresh gastric tissues were suspended in 125 ml solution of pH 2.2 containing 1% boric acid. pH of the solution was kept constant with O.1N HCl throughout the incubation period. Gastric tissues in acidic solution were placed at 40°C for 18 hr and it was stirred well occasionally. After this solution was filtered through cheese cloth and 200g^{-1} NaCl was added slowly with stirring the filtrate for complete precipitation of pepsin. The activity of dried crude pepsin was found to be 971 units mg^{-1} which can be further increased on purification.

References

1. V. Kastk, Scand. J. Clin. Labs. Invert. Suppl., **52,** 121 (1992).
2. J. H. Northrop, J. Gen. Physiol., **16,** 615 (1933).
3. R. M. Herriot; Q.M. Bartz and J. H. Northrop, J. Gen. Physiol, **21,** 575 (1938).
4. A. A. Olatunde, A. I. Ukoha, and B. F. Oguntayo Arch. Hydrobiol., **112,** 107 (1988).
5. I. Yu Sakharov and N. V. Sukhova, Izobreteniya, **12,** 128 (1992).
6. V. P. Degtyareva and T. V. Degtyareva, Dokl TSKHA, **265,** 42 (1981).
7. E. Valles and J. P. Furet, Lait, **61,** 590 (1981).
8. D. B. Emmons, B. Raiser, R. N. Girous and D. W. Stantle, Can. Inst. Food Sci. Technol., **9,** 189 (1976).
9. V. Husek and M. Debek, Neth. Milk Dairy J., **35,** 302 (1981).
10. B. Chorbanov, D. Kh. Bozhiva and V. Licher, Nahrung, **30,** 405 (1986).
11. E. Valles and J. P. Furet, Lait, **57,** 601 (1977).
12. R. M. Herriott and J. H. Northrop, Science, **83,** 469 (1936).
13. V. Kostka, Ira Pichora and M. Bandys, Proc. FEBS Adv. Course, 53 (1984).
14. T. Vladimir, J. Pohl, V. Kostka, G. Erdene, B. P. Tudarin and B. Dorzhpalam, Proc. FEBS Adv. Course 73 (1984).
15. T. Tageyama and K. Takohashi J. Biochem., **79,** 455 (1979).
16. M. L. Anson, J. Gen. Physiol., **22,** 79 (1988).
17. A. O. A. C. *Official Methods of Analysis* (Association of Analytical Chemists, Washington D.C., 1984).
18. E. Merck, *Handbook of Microbiology* (Darmstadt, Germany).
19. J. Antonini and B. R. Dumas, Biochimic **53,** 321 (1971).
20. T. Kageyama and K. Takahashi, J. Biochem., **80,** 983 (1976).
21. M. Djordjevic, B. Selivanovski, B. Mihajlovic; D. Dimitrijevic, M. Perovic and N. Gnjatovic, Technol. Mesa, **26,** 374 (1985).
22. T. N. Khangaldova, Izv Vyssh Uchobn Zaved Pishch Teknol, **5,** 152 (1975).