Optimisation of Activity and Storage Stability of Crude Pepsin Extracted from Adult Cattle Abomasa for Cheese Making

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Abstract

The study was conducted to investigate the activity and storage stability of crude pepsin extract for use as rennet substitute in cheese processing. The conditions tested were; regions of the abomasa, fresh versus dry abomasa; drying methods; maceration temperatures (37°C, 24-28°C or 1-5°C); maceration pH 1-6 for 6 days; activation pH (pH 1-4) and clarification by centrifugation or use of chemicals. The shelf life of the extract was evaluated under storage temperatures ranging from deep freezing at -20°C, refrigeration temperatures (<10°C) and room temperature. Results showed that, fundic region of the abomasa had 70.8% pepsin while the pyloric region had only 27% pepsin. Milk clotting activity (MCA) was 1978 and 1800 pepsin units per ml (PU/ml) for sun and shade-dried abomasa respectively. Concentrations of 5, 10, 15, 20, 25% NaCl gave 1845, 2189, 1596, 1021, 462 MCA respectively. Maceration at pH 3 for 3 days at 37 °C gave the highest enzymatic activity (5536 PU/ml). Clearing of the extract by centrifugation at 3000 g/15 min gave an extract with 902.3 PU/ml. Clarification by use of di-sodium phosphate (Na_2HSO_4) gave extract of 1679.1 PU/ml. The enzyme activity of the extract stored under deep freezer temperature was significantly higher ($P<0.05$) than ambient and refrigeration temperatures. However, they were significantly lower ($P<0.001$) than the activity of the extract before storage. Therefore, the best pepsin extraction conditions were found to be: the use of fundic region of sundried abomasa, macerated at 37°C in an extraction solution containing 5-10% salt at pH 3 for 3 days and clarified after activation at pH2 for 20 min followed by centrifugation of sediment at 3000g/15 min or by use of anhydrous disodium phosphate. The clarified extract could retain its activity for 16 weeks when stored at - 20°C in single use portions.

Keywords: Milk clotting activity, pepsin, rennet, abomasa, cheese.

Introduction

Calf rennet has traditionally been the main enzyme extract used in cheese making worldwide. With the increased demand for meat, however, calves are now allowed to reach maturity, thereby decreasing the supply of calf abomasa. Consequently, calf rennet has become less readily available and more expensive. This has stimulated the search for alternative milk coagulants or rennet substitutes.

In the search for a solution to this problem, experiments have been conducted worldwide in order to find an acceptable substitute for commercial rennet (Davide *et al.*1982). Proteolytic enzymes of microbial origin have been investigated. The microbial enzyme have not, however become generally accepted as they often lead to undesirable effect in many types of cheeses. The use of porcine pepsin from pig is used in some countries but in countries like Tanzania, is limited due to religious considerations. The substitution of rennin with bovine pepsin can fairly easily be carried out using abomasa from older cattle slaughtered in local abattoirs. Their availability and their relatively cheap price provide the best opportunity of being used as raw material for bovine pepsin extraction. Their advan-

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tage is the ease of preparation with a simple formulation of table salt and acetic acid in water and its applicability at the village level. Hagyard, (1972); Davide *et al.* (1982); Rand and Ernstrom, (1972) and Surrender & Dutta, (1980) have reported some of the extraction and storage conditions which affect the activity and yield of the crude extract which must be taken or avoided during the extraction processes. Earlier work at our laboratory by Wigenge, (1989) investigated the suitable method of extracting crude pepsin enzyme from cattle abomasa. Mugeni (1990) analysed the performance of the crude pepsin from cattle abomasa using modified method by Herrian *et al.* (1983). Their results showed that, fresh abomasa gave higher enzymatic activity when extracted under room temperature. Lyatuu (1991), analysed the optimisation of activity and storage stability of crude pepsin extraction from cattle abomasa. The results showed that the highest enzymatic activity can be obtained using 5 to 10% NaCl when the extraction process is carried out at physiological temperature. Due to the practical difficulties of handling fresh abomasa on a daily basis, it was considered desirable to further investigate the effect of the method of drying of cattle abomasa and other relevant extraction parameters on the milk clotting activity of pepsin extract from abomasa of adult cattle.

Materials and Methods

Fresh adult cattle abomasa were collected from a local town abattoir, opened and their content emptied, thoroughly washed with running water and the fatty tissue was trimmed off. The extraction solution was prepared according to the method described by Hagyard, (1972).

The MCA of the enzyme extract was determined by the Roll tube method according to IDF Standard 157:1992. Low heat dried skim milk powder was obtained from Tanzania Dairies Limited. Comparison of pepsin concentration in the fundic and pyloric regions of abomasa was done by using seven abomasa which were cut dorsally and sun dried (temperature 25-28°C) to constant weights which was attained in four days. The abomasa fundic and pyloric regions were easily distinguished by the presence of muscular tissues in the fundic and their absence in the pyloric regions. After separating these parts, each part was cut into small pieces (0.5-1 cm wide). Duplicate samples of 10 g were mixed with 100 ml extraction solution, macerated at room temperature (25-28°C) for 3 days. The crude extract was activated with 0.1 N HCl for 60 min and clarified by adding 1 g $Na_2HPO_4.H_2O$ for 20 ml extract to pH 5.4.

Comparison of pepsin activity from dried and fresh abomasa was done by using seven fundic regions of the abomasa which were slit into two equal portions. One part (127g) was dried under direct sunshine to constant weight of 30g. The amount of water lost was added to reconstitute the enzyme extract. Thirty grams of the sundried abomasa was cut into small cubes (0.5 cm), mixed with 97 ml water lost during drying plus 381 ml extraction solution with 0.76 g sodium benzoate. The other 127 g of fresh fundic abomasa was cut into pieces, ground in a warring blender, mixed with 381 ml extraction solution plus 0.76 g sodium benzoate. All the portions were macerated for the same period of 3 days, activated, clarified and the MCA determined.

Effect of drying methods of fundic region to the MCA, was done by using three fresh abomasa which were each divided dorsally into three parts and each part was dried to constant weight under direct sunshine (25-28oC), in the oven with forced air circulation (30oC) and under shade. Each part was cut into small pieces, duplicate samples of 10 g were mixed with 100 extraction solution, macerated, activated, clarified and MCA determined. Extracts were also determined for dry matter as described by Egan *et al.* (1981) and enumeration of total plate count (SPC) was done as described by IDF standard 100B:1991.

Determination of the optimum salt concentration for pepsin extraction was done by using dried pieces of fundic abomasa. Duplicate samples (10g) were mixed with 100 ml extraction solution of varying salt concentrations of: 5, 10, 15, 20 and 25 % NaCl. The samples were macerated for 3 days, activated, clarified and MCA determined.

Determination of optimum extraction temperature on MCA: Duplicate samples of dried pieces of fundic abomasa weighing 10 g were mixed with 100 ml extraction solution and macerated at 37 °C, room temperature of 24 -28 °C

and incubator temperature of 28-30 °C for 3 days. The extracts were activated, clarified and MCA was determined.

Determination of the optimum extraction time on MCA: Duplicate samples of 10g of dried pieces of fundic regions of the abomasa were mixed with 100 ml of extraction solution with pH ranging from 1 to 6. The samples were macerated for 6 days at room temperature, activated for 1 hour, clarified and MCA was determined at intervals of 1, 2, 3, 4, 5, 6 days.

Determination of the optimum activation pH and time on MCA: One hundred grams of dried pieces of abomasa were mixed with 1 litre of extraction solution. The mixture was macerated at room temperature for 3 days. Portions of 100 ml of the extract were activated to the pH of 1, 2, 3, and 4 respectively using 0.1 N HCl. When the activation pH was attained, 1 ml of the solution was drawn out at 10 min intervals and used to determine the MCA. This was continued until the MCA remained constant and the time taken to clot the milk was recorded as the optimum for activation.

The effect of clarification methods on MCA: After maceration and filtering, the crude extract still contained much fine tissues making it turbid and reduced the shelf life of the extract. Clarification therefore was necessary.

(i) Centrifugation: MCA of the crude extract was determined and then duplicate samples of 5 ml of crude extract were centrifuged at 1000 g for 10, 20 and 30min. This procedure was repeated for centrifugal speeds of 2000, 3000 and 4000g. The MCA of the clear solutions were then determined.

(ii) The remaining portion after step 1 of the macerated and filtered crude extract was clarified by adding solid forms of Na_2HPO_4, $Na_2HPO_2H_2O$, Na_2HCO_3 or NaOH in duplicate. These chemicals were added to the extracts gradually, stirred with magnetic stirrer until pH rose to 5.4 whereby the extract started to flocculate. The extract was left to settle and the MCA of the supernatant was determined.

Effect of different storage temperatures on MCA: Duplicate samples of clarified pepsin extracts were stored at three temperature regime: room temperature (25-28°C), refrigeration (0-5°C and freezer temperature (-18 to -20° C). MCA was determined before and after 4 weeks of storage.

The effect of interaction of different extraction conditions

Ten gm of sun dried pieces of abomasa were put into 108 flasks of 250 ml and 100 ml of extraction solution with pH 1, 2, 3, 4, 5 and 6 to which NaCl concentration levels of 0, 10, 20 % were added. Duplicate sample of each pH and its three NaCl levels were macerated at three temperature regimes of 0-5 0C, 24 – 28 oC and 37 oC for six days. At day 1, 3 and 6 crude extract of 20 ml was drawn from each flask, filtered, centrifuged and MCA was determined. Design used was: 6 pH x 3 NaCl x 3 temperature x 3 days (6 x 3 x 3 x 3 factorial design).

The statistical model :

$$Y_{ijklm} = \mu + P_i + S_j + T_k + D_l + (PS)_{ij} + (PT)_{ik} + (ST)_{jk} + E_{ijkl}$$

Y_{ijklm} = mth measurement from the l th day of the k th temperature, j th salt concentration and i th pH;

μ = general mean common to all measurements;

P_i = effect of the i th pH level;

S_j = effect of j th salt concentration;

T_k = effect of i th temperature;

D_l = effect of l th day of maceration;

$(PS)_{ij}$ = interaction between the i th pH and j th salt concentration;

$(PT)_{ik}$ = interaction between the i th pH and the k th temperature;

$(ST)_{jk}$ = interaction between the j th salt concentration and the k th temperature

E_{ijklm} = random error peculiar to each observation

Results and discussion

Results showed that pepsin concentration in the fundic and pyloric regions of the abomasa were significantly different ($P>0.001$) containing 1.08mg/ml and 0.46mg/ml or 72 and 27% pepsin respectively (Table1). This showed that pepsin concentration in fundic region was about 2-3 times higher than in the pyloric region. The total pepsin concentration was within the range reported by Andren *et al.* (1981) who reported 1-2 mg/ml. Andren, (1992) reported that the

differences of the bovine pepsin in the two regions was obvious due to the anatomical/histological differences existing in the abomasum. Therefore only the fundic region should be used for pepsin extraction.

Table 1: Protein concentratin (mg/ml) and percentage of pepsin in the fundic and pyloric region of the abomasa(N=14)

Amount pepsin	Protein (mg/ml) Lsmeans (±se)	%pepsin	Pr>T
Fundic	1.08 ± 0.05[a]	72.2	0.001***
Pyloric	0.46 ± 0.05[b]	27.4	

Key: 1- Number of observations *** Highly significantly different

Means within column followed by similar superscript letters do not differ significantly (P>0.05) according to Duncan multiple range test (DMRT)

Results on the effect of extracting pepsin from dried versus fresh abomasa (Table 2) showed that extracts from dried fundic region gave higher MCA (3296 PU/ml) than from the fresh material (2326 PU/ml). This results agree with that reported by Hewedy *et al.* (1992). They obtained higher MCA in milled, dried or sliced dried tissue than in fresh minced calf tissue and reported that drying modifies the mucus nature of the abomasa which then resulted in high MCA of the extracts. Also Hagyard, (1972) showed that dried veils increased the yield of MCA by 11% over that obtained from fresh veil materials. In this study MCA of 29.4% higher was obtained in dried compared to fresh extracts from the fundic region of the abomasa.

Table 2: Milk clotting activity (PU/ml) from extracting fresh versus sun dried abomasa (N=14)

State abomasa	Protein(mg/ml) Lsmeans (± se)	Pr >T
Dry	3296 ± 11[a]	0.001***
Fresh	2326 ± 11[b]	

Key: 1- Number of observations ***Highly significantly different

Means within a column followed by similar superscript letters do not differ significantly(P>0.05)according to DMRT.

Table 3 shows that MCA was higher in sun dried fundic extract than in the shade and oven dried. The results agree with findings by Hagyard (1972) who reported that sun-shine or sun rays reduce the viscous or mucus nature of the abomasa tissue and the ratio of the various zymogens present in the pro-rennin and pepsinogen so that during the enzyme extraction process there is an increase in yield and a favoured conversion of prorennin or pepsinogen. The effect of direct sun drying on the abomasa also explains why the dry matter percentage in the sundried abomasa extract was lower than extract from abomasa dried under the shade (Table3). This could be because the un-modified mucus decomposed during long drying of the abomasa and were included in the extracts during pepsin extraction hence increasing the dry matter. The microbial quality showed no significant difference (P>0.05) between the two drying methods. However, the calculated mean values of 2.3 x 10^1 cfu/ml of extract from sundried abomasa was lower than that for shade dried extract (2.7 x 10^4 cfu/ml) implying better microbial quality of crude extract obtained by sun drying. The study agrees with the report by Hagyard. (1972) who indicated that sundrying preserves the enzyme through fast drying and possibly the germicidal effect of direct sunrays. On account of this slight advantage, sundrying may therefore be employed in drying of cattle abomasa for bovine pepsin extraction.

Table 3: Effect of drying abomasa under sunlight and unde the shade on MCA, dry matter and bacterial count (N=6)

Drying methods	Paramenters determined			Pr>F
	Pepsin (PU/ml)	% Dry matter	SPC(cfu/ml)	
Direct Sun (25-30°C)	1987±19[a]	15.73±19[a]	2.3x10^4±19[a]	0.009***
Shade (25-30°C)	1800±14[b]	17.30±14[b]	2.7x10^4±14[b]	0.001***
Oven (30°C)	1609±33[c]	ND	ND	0.033**

Key: ND - Not done N-Number of observations
** Significantly (P>0.01) *** Highly significatly different (P>0.001)

Means within a column followed by similar supercript letters do not differ significantly (P>0.05) according to DMRT.

Figure 1 shows a positive relationship between salt concentration and MCA of the extract up to 10 % salt and negative effect on MCA when concentration is more than 10% salt. These findings are in agreement with those of Rand and Ernstron, (1972); Whitaker, (1972); O'Leary and Fox, (1975); Vesitera and Tsyrenova, (1985) and Lyatuu, (1991) who reported that yield and MCA increased by increasing salt concentration between 5-10 % salt. Whitaker, (1972) reported that low and high salt concentration would normally reduce the ability of the extraction solution to de-sorb the enzyme from its attachments. Moreover, Anis *et al.* (1983) reported that the direct relationship between salt concentration and MCA was due to the phenomena and theory of partial salting out of the enzyme as well as the cation exchange taking place between added salt and calcium ions of milk which result in extended clotting time.

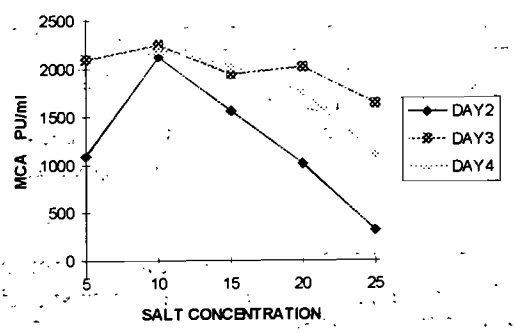
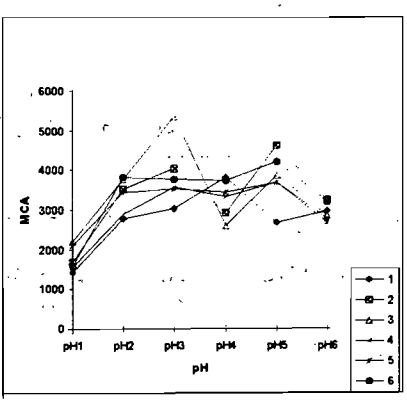


Figure 1: Effect of NaCl concentration and extraction time on MCA (PU/ml) of the sun dried fundic abomasa

Results of the determination of optimum extraction temperature show that the extraction temperatures of 37°C, 28- 32°C and 0 - 5°C lead to significant differences in MCA ($P<0.05$) of 2727, 1888 and 332 PU/ml respectively. Herrian *et al.* (1983) noted that 37°C which is the temperature in the stomach of the cow, is in favour of crude pepsin activation because the conversion of inactive pepsinogen to active pepsin is taking place under normal physiological conditions. Other optimum temperatures for pepsin extraction have been reported to be 37-42°C by Nelson, (1975); 28- 32°C by Davide *et al.* (1982); Peralta *et al.* (1986) and 40-42°C by Herrian *et al.* (1983). The high temperatures used in these studies normally required available electricity and maintenance of incubator temperatures for pepsin maceration. Under Tanzanian conditions, however, extraction at 25-30°C is most appropriate because it is the temperature found in most parts of the country. Small scale cheese processors will not need to use incubators but would take advantage of the prevailing high ambient temperatures throughout the year to extract bovine pepsin. In colleges, universities and processing plants where incubators are available, 37°C is recommended for extraction.

Figure 2 show that optimum extraction time and pH was obtained when the dried abomasa was macerated at pH 3 for 3 days. This result disagrees with findings by Hewedy *et al.* (1992) who reported that MCA of calves and adult cattle abomasa had a gradual increase in activity up to 8 days then decreased on the 10th day. The differences in findings between the present study and those of Hewedy *et al.* (1992) could be due to variations in temperatures, pH, salt content and then of pepsinogen into pepsin.

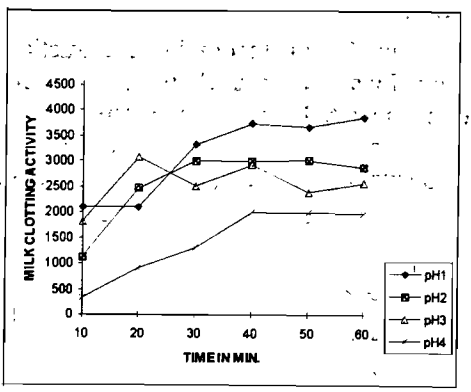


Key 1,2,3,4,5,6. Extraction period (days) of the fundic abomasa)

Figure 2: Effect of extraction time and pH on MCA of crude extracts from the sun dried fundic abomasa

Results (Figure 3) show that conversion of pepsinogen to pepsin increased as pH decreased with highest MCA at pH 1, but the enzyme obtained at this pH was not stable. This was in agreement with the findings of Rajagopalan, *et al.* (1966) who indicated that it was due to the formation of active species when activation pH was low. Similar results were reported by Harboe *et al.* (1974) who found out that when

pepsinogen is activated at pH 2 for a minimum time of 20 min full conversion to pepsin is achieved. Foltmann, (1993) reported the non-covalent intermediates between prosegment of peptides and pepsin and isolated complexes of Leu1p-Leu44p/pepsin and Leu1p-Leu16p/ pepsin formation during long time activation of pepsin. This agrees with the present study in which MCA increased after activation at different pH used, but prosegment peptides were not isolated



Key: pH 1,2,3,4 activation pH levels of crude pepsin extracts

Figure 3: Effect of activation time and pH o MCA of the crude pepsin extracts of the fundic abomasa (macerated for 3 days at pH 3)

Results on the effect of clarification methods (Table 4) show that centrifugation speeds on the crude extracts was not significantly different (P>0.05). Despite the lack of significant difference, in mean separation, there were slight differences where 3000 g for 15 min gave the highest MCA while 1000 g for 15 min gave the lowest MCA. Also there were no significant differences between centrifugation and other physical separation of abomasal tissue (P>0.05) (Table 4), although a centrifugation speed of 3000 g for 15 min and gravitational separation of chemically activated tissue gave higher MCA than others. Filtering gave clearer extracts than other methods but a lot of time was used to filter the extracts. $Na_2HPO_2.H_2O$ and Na_2HPO_4 were more efficient in separating the abomasal tissue from the enzyme than other chemicals (Table 4). Furthermore, of the other chemicals used in this study, NaOH is corrosive and requires more care in handling and Na_2CO_3 gives a lot of foam due to CO_2 production during clarification. Therefore the use of $Na_2HPO_2.H_2O$ and Na_2HPO_4 for clarification are recommended for small scale cheese producers.

Table 4: Effect of crude extract clarification methods on MCA.

Treatment	N	Time (min)	Chemicals	N	Milk Clotting time (min)
No treatment	20	2.00	No treatment	12	6.00[a]
Gravitational sedimentation	20	1.13	$Na_2HPO_4.H_2$	12	2.98[c]
Centifugation	20	1.20	Na_2HPO_4	12	3.10[c]
Filtration	20	1.25	NaOH	12	4:30[b]
			Na_2CO_3	12	3.45[b]

Means within a column followed by similar superscript letters do not differ significantly (P>0.05)according to DMRT.

Centrifugation Speed on MCA

G/15 min	N	1000	2000	3000	4000
MCA PU/ml	8	860 ± 13^a	889 ± 9^a	902 ± 10^a	899 ± 5^a

Key: N-Number of observations

Means of PU/ml within a row followed by similar superscript letters do not differ significantly according to DMRT.

Effect of different storage temperature regimes (Table 5) show that there was no significant difference (P>0.05) between MCA of extracts stored at ambient temperature and those at refrigeration temperature. MCA of the extract stored in the deep freezer and the freshly prepared extract differed significantly (P<0.001) from the other two treatments. MCA of the extracts stored at deep freezing temperature gave the highest enzymatic activity as compared to those kept under ambient or refrigeration temperatures. Similar findings were reported by Kassell and Meitner, (1970) who reccommended that pepsinogen solutions be stored at -200C. They reported that enzymes are very sensitive to incidental activation by acid, denaturation and bacterial contamination which could lower the activity of extracts stored at room temperatures as seen in this study. However, they suggested that crude extracts must be stored in portions that can be used at once to avoid re-thawing and recontamination by microbes.

Table 5: Effect on MCA of different storage temperature regimes of the clarified extracts for weeks (N=6)

Storage temperature	Pepsin units/ml (LSMeans)	Standard error
Initial	562 [c]	97
Room	1049 [b]	97
Fridge	1030 [b]	97
Freezer	13767 [a]	97

Key: N-Number of observations

Means of PU/ml within a column followed by similar superscript letters do not differ significantly according to Duncan's multiple range test

Conclusions and recommendations

From this study it can be concluded that the extraction conditions which led to optimum MCA of bovine pepsin preparations from adult cattle abomasa were:- the use of the fundic region of the abomasa, extracting pepsin from sun dried fundic region of the abomasa, maceration of the dried abomasa at pH 3 for 3 days, use of 10% NaCl in the extraction solution, extraction temperature of 37 o C for 3 days using an incubator or room temperature (25 -30 oC), activating the crude extract at pH 2 for 30 min and clearing the crude extract by addition of $Na_2HPO_4.H_2O$ and centrifugation at 3000g for 15min or gravitational sedimentation.

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