FACTORS AFFECTING THE EXTRACTION OF PROTEASE FROM JAWASEE (*ALHAGI PSEUDALHGI (DIEB) DESYSYN A CAMELORUMFISCH*)

K. Yeshodha, S. C. Dhar & M. Santappa

Central Leather Research Institute, Madras.

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Various experimental procedures for the extraction of protease from jawasee powder were adopted to ascertain optimum conditions for leaching. Deionized distilled water at pH 7.0 and 37°C was found to extract maximum enzyme in 24 hours. The jawasee protease is optimally active at pH 6.0 with 2.5% egg albumin as substrate. Sodium chloride at higher concentration inhibits the enzyme considerably. Strong inhibitory action was observed in case of 4 preservatives, namely, *para*-chloro-*meta*-cresol, sodium pentachlorophenate, phenyl mercuric nitrate and sodium trichlorophenate

Introduction

Proteolytic enzymes have wide application in the fields of brewery, textile, pharmaceutical and leather industries. In the leather industry, enzymes are used during the process of unhairing and bating of skins and hides. These enzymes can be derived from animal, microbial or plant sources. Since the process of liming has a detrimental effect on keratin, it leads to loss of quality wool/hair which forms a valuable by-product. The enzymic process of unhairing results in the recovery of wool/hair without causing any damage to either wool or pelt.

The shrub jawasee, abundantly available in Rajasthan and Gujarat, is used as an unhairing agent there. Although a few leather manufacturing processes have been described[1-5], the exact role of enzyme in the unhairing process is not yet known. In a recent publication[6], jawasee shrub has been described as a potent source of enzymes which could be successfully used in unhairing hides and skins. Studies have also shown that the unhairing of hides and skins is due to the proteolytic enzyme present in the leaves and bark of the shrub. Since jawasee is found to be a rich source of a protease, it was thought desirable to study the physico-chemical properties of the enzyme for which extraction and isolation of enzyme from the shrub was made.

The object of the present investigation is to study various optimum conditions to get maximum amount of enzyme extract which can be used for the purification of this enzyme.

Materials and Methods

The jawasee along with leaves was dried in shade and powdered. Fresh powder was

obtained from Gujarat and Rajasthan, sieved and the fine powder was used for this investigation.

Extraction of crude enzyme

Weighed quantities of jawasee powder were allowed to be leached at 37°C for various time intervals in water as well as in buffer with occasional stirring. The enzyme extract was separated first by filtering through filter cloth and then by centrifuging the filtrate. The supernatant was used as the crude enzyme extract.

Enzyme assay

For estimating the proteolytic activity of the enzyme quantitatively, Anson's Colorimetric method[10] of estimating the liberated tyrosine during proteolysis, as adopted by Bose *et al.*[11] was followed with slight modification. To state briefly, 5 ml. aliquot of enzyme extract was added to 10 ml. of 2.5% egg albumin solution (pH 6.0) and the mixture was allowed to be digested at 45°C for 30 minutes. After the period, 30 ml. of 5% trichloro acetic acid was added to stop the enzyme action, warmed on a boiling water bath, cooled to room temperature and filtered through whatman No. 3 filter paper. To 2.5 ml. of the filtrate were added 5 ml. of 0.5 N sodium hydroxide and 1.5 ml. of twice diluted phenol reagent. The mixture was whirled for exactly two minutes and the blue colour so developed was measured in the Klett-Summerson photo-electric colorimeter at 660 nm. A control was always run in an identical manner except that the trichloroacetic acid solution was first added to the egg albumin solution and then the enzyme extract was added to the mixture. The proteolytic activity is expressed as unit of enzyme activity which is defined as the amount of enzyme that liberates one microgram of tyrosine from egg albumin at pH 6.0 and 45°C in 30 minutes.

Results

In order to obtain maximum amount of enzyme in the crude enzyme extract from the jawasee powder, various alternative conditions were tried for leaching out the enzyme from the powder.

Effect of different buffers on the leaching characteristics of jawasee enzyme

0.5 g portions of jawasee powder were added to 50 ml. each of veronal or phosphate buffers (pH 6.0) in 4 glass stoppered bottles. One bottle containing veronal and one bottle containing phosphate buffers were incubated for 2 hrs. and the other two bottles were incubated for 24 hrs at 37°C with occasional shaking. After the periods, the insolubles were removed by filtration and the filtrates were analysed for proteolytic activity as described earlier. The enzyme activity is calculated as enzyme unit and the results are presented in Table I.

TABLE I

Effect of buffers

Buffer	*Proteolytic activity in enzyme units*	
	2 hours	*24 hours*
Veronal	28.8	115.2
Phosphate	80.2	298.8

Results indicate that phosphate buffer gives a better leaching of the enzyme than veronal buffer.

Effect of different periods of leaching in buffer on the proteolytic activity

Since phosphate buffer was found to produce effective leaching of the enzyme,

it was desired to study different periods of leaching in phosphate buffer to effect maximum leaching of the proteolytic enzyme. To 100 ml. of phosphate buffer (pH 6.0) 1 g. of jawasee was added in glass stoppered bottles and incubated at 37°C for 48 hours with occasional shaking. At regular intervals, the solutions were taken out, filtered and the filtrates were analysed for proteolytic activity. Results are presented in Fig. 1A.

It can be seen from Fig. 1A that maximum leaching of the proteolytic enzyme occurred on 24 hours incubation and further incubation led to minor increase in enzymic activity.

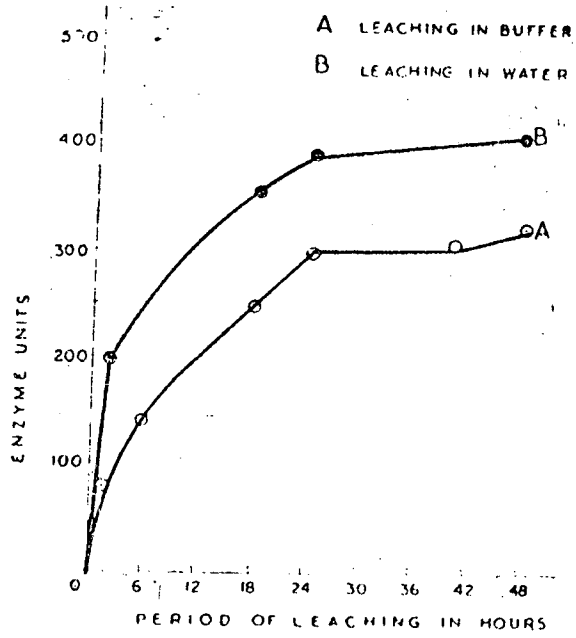


Fig. 1 : Leaching of enzyme in buffer and water

Effect of different periods of leaching in water on the proteolytic activity

In order to find out the efficiency of leaching enzymes in deionized distilled water, 1 g. portions of jawasee powder were mixed with each of 100 ml. deionized distilled water (pH 6.0) and the activity of enzyme extract was studied in the same manner as described in the case of phosphate buffer. The results are presented in Fig. 1B.

As can be seen from Fig. 1B, leaching for different periods in deionized distilled water resulted in a gradual increase in proteolytic activity upto a period of 24 hours when maximum leaching of the proteolytic enzyme from the jawasee powder was observed. There is however a slight increase in activity when kept for 48 hours. It was, therefore, decided to maintain a leaching period of 24 hours in deionized distilled water in course of subsequent experiments.

Effect of pH on the leaching characteristics of the protease

In order to find out the pH at which maximum leaching of the enzyme occurred, the effect of pH on the leaching characteristics of the enzyme was studied. 1 g. portions of jawasee powder was mixed with 100 ml. portions of deionized distilled water of different pH values in glass stoppered bottles and kept at 37°C for 24 hours. After the period, enzyme extracts were collected by filtration and filtrates were analysed for proteolytic activity as described earlier. The results are presented in Fig. 2A.

From Fig. 2A, it can be seen that maximum leaching of the enzyme occurred at pH 7.0.

Effect of leaching temperature on the activity of the protease

1 g. portions of jawasee powder were mixed with 100 ml. portions of deionized distilled water (pH 7.0) in different glass

stoppered bottles and incubated at different temperatures for 24 hours with occasional shaking. After the period, the enzyme extracts were collected by filtration and the filtrates were analysed for proteolytic activity. Results are presented in Table 2.

TABLE 2

Temperature – Activity relationship

Temperature in °C	*Proteolytic activity in enzyme units*
10	126.03
25	226.8
32	306.0
37	597.6
45	568.8

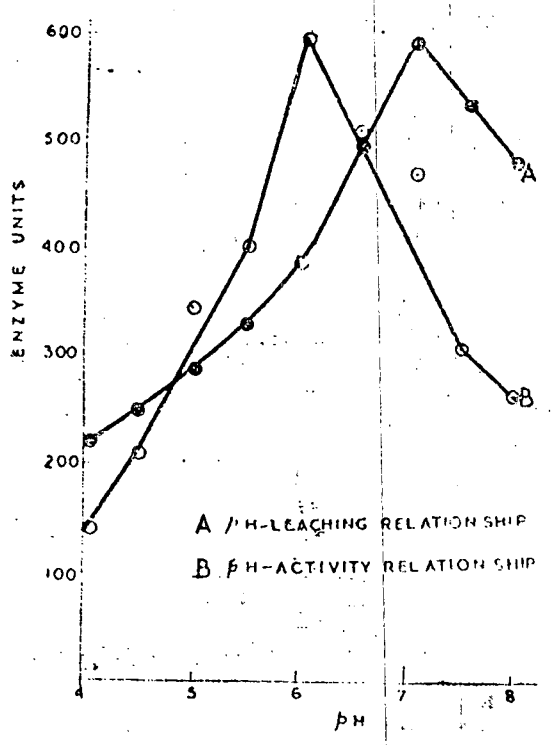


Fig. 2: Effects of pH on leaching and enzyme activity

From Table 2, it can be seen that leaching of the enzyme was maximum at 37°C and hence, this temperature was maintained for the extraction of the enzyme in all subsequent experiments.

Extraction of protease as influenced by the concentration of jawasee powder

In order to see whether addition of higher percentage of jawasee powder in water would in any way affect the extraction of enzyme, increasing concentrations (0.5-4.0%) of jawasee powder were used in the same manner as in the case of 1% powder. The proteolytic activity for each concentration was studied and presented in Fig. 3.

It can be seen from Fig. 3 that 1% concentration of jawasee powder was most suitable for maximum leaching of the proteolytic enzyme at pH 7.0 and 37°C for a period of 24 hours.

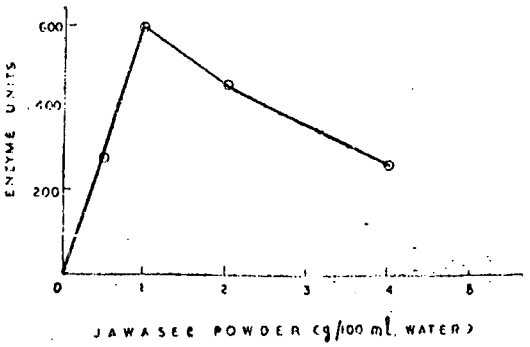


Fig. 3: Concentration of jawasee powder –activity relationship

Effect of substrate concentration on the activity of the protease

The effect of different concentrations of the substrate on the proteolytic activity of the jawasee enzyme was studied in order to find out the optimum concentration of the substrate required for maximum activity of the enzyme. 1 g. of jawasee powder in 100 ml. of deionized distilled water at pH

7.0 was incubated at 37°C for 24 hours. The solution was filtered after incubation and the filtrate was added to the different concentrations of substrate. The enzyme activity for each substrate concentration was determined as described earlier. Results are presented in Fig. 4.

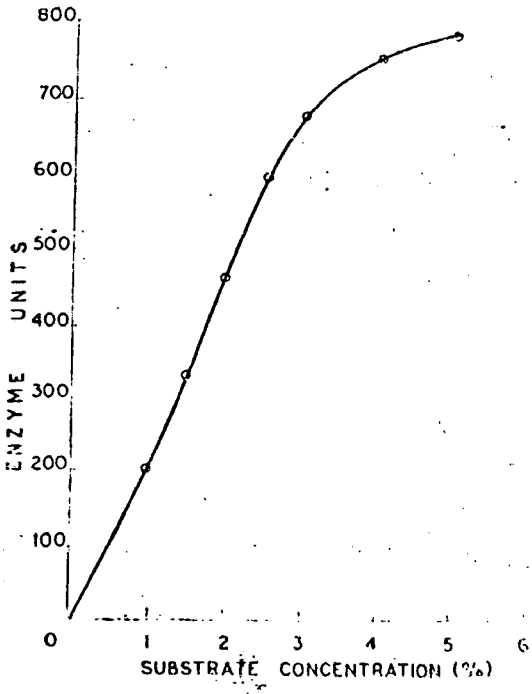


Fig. 4 : Substrate concentration —activity relationship

Results indicate that 2.5% egg albumin solution formed the optimum substrate concentration for maximum hydrolysis by the proteolytic enzyme of jawasee. Hence, this substrate concentration was followed throughout the present investigation.

Effect of pH on the activity of the protease

After establishing the optimum conditions for the extraction of enzyme from jawasee powder, it was thought essential to study the effect of pH on the activity of the protease in order to find out the pH at which the jawasee exhibited maximum proteolytic activity. 1 g. of jawasee powder in deionized distilled water at pH 7.0 was incubated at 37°C for 24 hours and the enzyme extract was collected by filtration. The filtrate was used as the crude enzyme extract. Equal volumes of 5% egg albumin solution were mixed with equal volumes of phosphate buffer of different pH values. The solutions thus obtained were mixed with crude enzyme extract and incubated at 45°C for 30 minutes. The enzyme activity in each case was determined as described earlier. The results are presented in Fig. 2B.

From Fig. 2B, it can be seen that the proteolytic activity of jawasee was maximum at pH 6.0, when egg albumin was used as the substrate.

Effect of various concentrations of sodium chloride on the proteolytic activity of jawasee

Since sodium chloride is normally used in the jawasee process of unhairing, it was thought necessary to study the effect of various concentrations of sodium chloride on the leaching and proteolytic activity of the jawasee enzyme. 1 g. portions of jawasee powder were taken in 100 ml. of deionized distilled water at pH 7.0 in different glass stoppered bottles and were incubated at 37°C for 24 hours with various concentrations of sodium chloride. After the period, the enzyme extracts from each bottle were separated by filtration and the filtrates were analysed for the enzyme activity. Results are presented in Fig. 5.

From Fig. 5, it can be seen that sodium chloride in all concentrations has inhibitory effect on the enzyme although in different degrees.

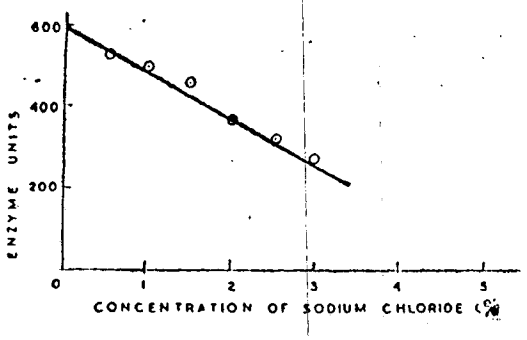


Fig. 5: Effect of sodium chloride on enzyme activity

Effect of sodium pentachlorophenate on the proteolytic activity of jawasee

In order to study the effect of sodium pentachlorophenate on the activity of the proteolytic enzyme, various concentrations of sodium pentachlorophenate (0.002–0.02%) were mixed with 1 g. portions of jawasee powder in 100 ml. portions of deionized distilled water (pH 7.0) and incubated at 37°C for 24 hours. At the end of the incubation period, the solutions were collected by filtration and the activity of the filtrates were tested by the usual procedure. Results are presented in Table 3.

TABLE 3

Effect of sodium pentachlorophenate on the activity of jawasee enzyme

Sodium pentachlorophenate (%)	*Proteolytic activity in enzyme units*
Control	597.6
0.002	589.1
0.006	580.9
0.01	575.7
0.014	574.4
0.02	564.1

From Table 3, it can be seen that small concentrations of sodium pentachlorophenate do not cause any significant reduction in the leaching and proteolytic activity of the jawasee enzyme.

Effect of preservatives

During the course of unhairing of hides and skins by enzyme, certain preservatives are used to prevent bacterial damage of skin protein; it was, therefore, thought worthwhile to study the effect of these preservatives on the proteolytic activity of the jawasee enzyme. In order to study the effectiveness of the preservatives, different concentrations of preservatives and different periods of incubation were investigated. 1 g. portions of jawasee powder were mixed with 100 ml. portions of deionized distilled water (pH 7.0) in glass stoppered bottles and different concentrations of preservatives in solid form were added to the bottles which were then incubated for 2 hours and 24 hours at 37°C. The enzyme extract from each bottle was collected by filtration and estimated for the proteolytic activity as described earlier. The results are presented in Table 4 and Fig. 6.

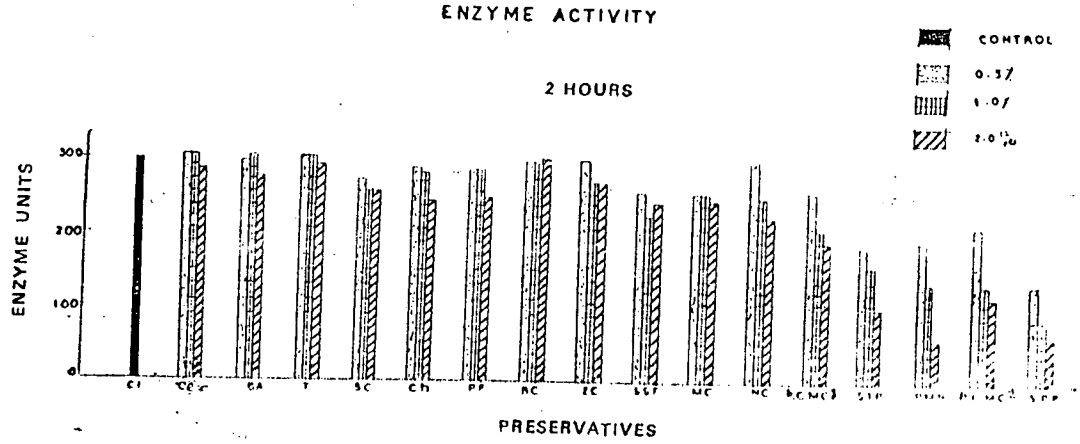
Discussion

In order to extract the enzyme from jawasee powder, 2 buffer systems, namely, veronal and phosphate buffers (pH 6.0) were tried for 2 hours and 24 hours. Although in both the cases, 24 hours leaching period showed higher enzymic activity, phosphate buffer was found to be more efficient than veronal buffer (Table 1).

A gradual increase in proteolytic activity in the enzyme extract was observed when leaching in phosphate buffer (pH 6.0) was extended upto 48 hours (Fig. 1A). The same trend of result was also observed when

EFFECT OF PRESERVATIVES ON ENZYME ACTIVITY

FIG 6



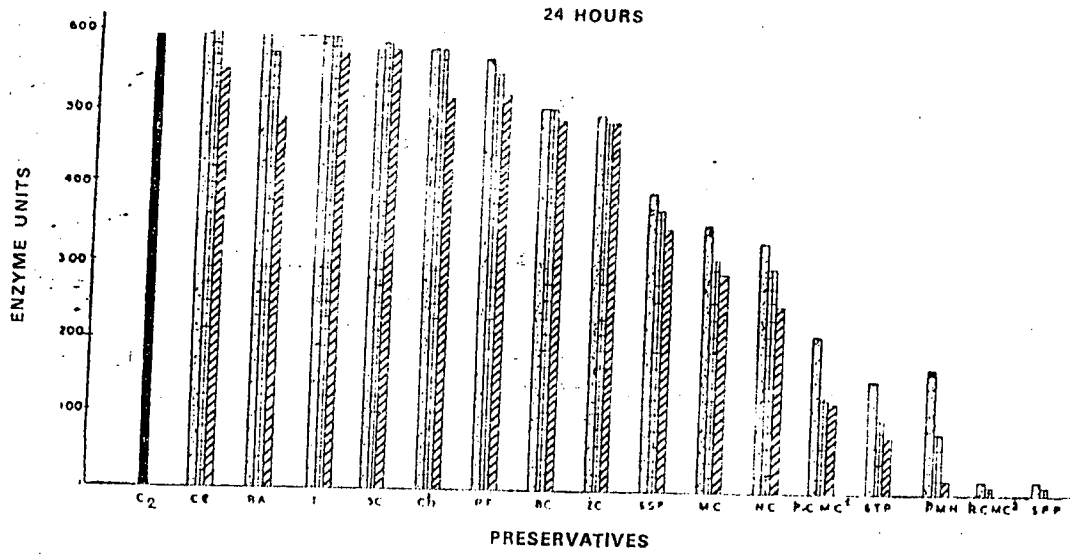


TABLE 4

Effect of preservatives on enzyme activity during leaching

No.	Preservatives	Period of leaching					
		2 hours			24 hours		
		Concentration of preservatives					
		0.5%	1%	2%	0.5%	1%	2%
1.	Control 2 hours : 294.4 24 hours : 597.6						
2.	Cetrimide (BP) (Ce)	299.8	299.8	285.2	604.8	602.2	554.7
3.	Boric acid (BA)	298.1	299.6	271.8	600.1	576.8	491.2
4.	Toluene (T)	300.2	300.1	292.6	600.3	571.9	590.9
5.	Sodium chlorite (SC)	270.0	254.7	254.6	580.2	586.8	579.8
6.	Chloroform (Ch)	285.7	280.4	240.8	583.1	583.1	515.8
7.	Potassium fluoride (PF)	285.6	285.5	275.2	571.3	550.2	524.4
8.	Benzalkonium chloride (BC)	295.2	295.4	300.1	509.8	507.3	490.3
9.	Zinc chloride (ZC)	294.9	249.9	268.8	500.2	474.8	485.3
10.	Sodium silico fluoride (SSF)	256.1	216.8	234.2	398.2	374.7	346.3
11.	Mercuric chloride (MC)	250.0	250.0	239.2	355.9	310.1	286.9
12.	Nickel chloride (NC)	297.8	242.2	220.4	333.3	300.2	247.7
13.	*Para*-chloro-*meta*-cresol* (in water) (p-CMC[1])	257.1	202.4	183.8	210.1	128.4	120.3
14.	Sodium trichlorophenate (STP)	177.8	152.2	100.3	150.4	100.2	75.4
15.	Phenyl mercuric nitrate (PMN)	188.4	132.7	58.4	164.7	82.7	23.3
16.	*Para*-chloro-*meta*-cresol* (in alcohol & water) (pCMC[2])	207.8	130.1	112.7	20.4	9.8	0.0
17.	Sodium pentachlorophenate (SPP)	133.4	86.2	56.9	20.3	9.8	0.0

*Since *para*-chloro-*meta*-cresol was found to be not fully soluble in water, it was first tested in aqueous medium. In subsequent experiment, however, *para*-chloro-*meta*-cresol was first dissolved in minimum quantity of alcohol, made up the volume with water and then used.

leaching was carried out with deionized distilled water at pH 6.0 (Fig. 1B). Leaching with water, however, was found to extract more enzyme than phosphate buffer under the same experimental conditions.

It can be seen from Fig. 2A that the maximum enzyme is extracted at pH 7.0 and 37°C for 24 hours.

At lower temperature of leaching, enzyme was not found to be extracted optimally. At higher temperature (45°C), loss in enzyme activity was observed, optimum temperature of leaching being 37°C (Table 2).

Fig. 3 indicates that 1 g. of jawasee powder in 100 ml. deionized distilled water at pH 7.0 gave maximum enzyme activity in the extracted liquor. High concentration of jawasee powder did not give higher yield of enzyme activity.

When same concentration of enzyme was added to different concentrations of the substrate (egg albumin), it was observed that enzyme-substrate linearship is maintained upto a concentration of 2.5% egg albumin solution (Fig. 4).

The results presented in Fig. 2B indicate that maximum enzyme activity was found when the digestion was carried out at pH 6.0. This observation is in full agreement with that of Madhavakrishna and George,[3] who found pH 6.0 as the optimum pH for unhairing by jawasee process.

Results presented in Fig. 5 indicate that as there is gradual increase in the concentration of sodium chloride, there is gradual decrease in enzymic activity. This is quite evident from the fact that in the actual process of unhairing, loosening of hair is very much delayed due to the presence of sodium chloride in the enzyme bath. In the actual unhairing process salt is used to avoid the risk of damages due to bacterial action, but the unhairing period is prolonged from 6 to 20 days, depending on the amount of salt and quantity of jawasee powder used.[4] Normally unhairing with jawasee and salt was found to take 15 to 20 days, whereas it was possible to unhair within 48 hours with jawasee alone.[3]

In actual practice jawasee process of unhairing takes considerably long period resulting in foul smell due to bacterial action. In order to avoid the bacterial damage, sodium chloride as high as 10% is used. It was observed that sodium chloride at higher concentrations was found to inhibit the enzyme to a large extent (Fig. 5). Hence, use of sodium chloride as preservative is not recommended. A large number of preservatives in various concentrations were tried. Sodium pentachlorophenate, which is a strong preservative for hides and skins was found to have no effect on the activity of the enzyme at low concentrations upto 0.02% (Table 3.) Out of the 16 preservatives tried, only 4 preservatives, namely, *para*-chloro-*meta*-cresol, sodium pentachlorophenate, phenyl mercuric nitrate and sodium trichlorophenate showed strong inhibitory action on enzymic activity. It was observed that 2 hours of leaching in the presence of cetrimide, benzalkonium chloride, toluene, sodium chlorite, chloroform, potassium fluoride, boric acid, zinc chloride, nickel chloride, sodium silicofluoride and mercuric chloride did not result in significant reduction in enzyme activity upto 2% concentration of the preservative. *Para*-chloro-*meta*-cresol, sodium trichlorophenate, phenyl mercuric nitrate and sodium pentachlorophenate were found to considerably reduce the proteolytic activity of jawasee, even in 0.5% concentration on leaching for as short a period as 2 hours.

Leaching for a period of 24 hours in the presence of cetrimide, boric acid, formic acid, sodium chloride, chloroform and potassium fluoride did not affect the proteolytic activity appreciably. Boric acid and zinc chloride were found to produce negligible reduction in activity while sodium silicofluoride, mercuric chloride and nickel chloride were found to cause considerable inhibition; *para*-chloro-*meta*-cresol, sodium trichlorophenate, phenyl mercuric nitrate and sodium pentachlorophenate were found to be inhibitory, *para*-chloro-*meta*-cresol (in alcohol added water) and sodium pentachlorophenate causing almost complete inhibition of proteulytic activity. (Table 4 and Fig. 6).

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