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Three Phase Purification of Milk Clotting Protease from Wrightia tinctoria Fruit and Studies on Its Casein Subunit Specificity

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Abstract

Crude aqueous extract of Wrightia tinctoria fruit was subjected to three phase partitioning (TPP) with three different ammonium sulphate concentrations (40, 60, 80%) and their effect on concentrating milk clotting proteases were examined with 1:1 ratio of crude extract to t-Butanol. Proteases that concentrated in the interphase (IP) fraction of 60 and 80% salt concentration exhibited high milk clotting activity. TPP contributed to reduction in Caseinolytic Activity (CA) and enhancement of Milk Clotting Index (MCI) in these IP fractions compared to the crude enzyme. Casein (whole and kappa) hydrolytic pattern was analyzed by tricine SDS PAGE. Electrophoretic pattern of hydrolysate after 1 hr incubation revealed hydrolysis of κ-CN by CE, IP 60 and IP 80 fractions. Appearance of two low molecular weight bands approximately around 15 kDa indicated the specific affinity and controlled hydrolytic behavior by the proteases. Study provides evidence towards the utility of TPP in concentrating these milk clotting proteases and in improving their MCI.

Index Terms

Cheese, Milk Clotting, Proteases, Wrightia tinctoria.

INTRODUCTION

Cheese is a popular dairy product derived from milk, valued for its nutritional content. The method of preparation of cheese varies greatly according to the region, climate and different cultures. Coagulation of milk followed by pressing and salting are the basic steps in cheese preparation. Animal rennet, used in milk coagulation, is obtained from abomasum of unweaved calves. The demand for calf rennet has escalated over the past decades but supply has not met the level demanded. Moreover, sourcing of rennet by slaughtering of animals gives rise to various ethical issues and also religious restrictions oppose consumption of animal derived products. In order to meet the escalating demand and address ethical issues, plant coagulants have been explored as substitutes. Plant proteases have been used in traditional cheese making in various parts of the world. Based on this traditional knowledge, many plants have been screened for their milk clotting potential. One such plant is *Wrightia tinctoria*, which has been used in traditional cheese making and recent studies have isolated and partially purified milk clotting proteases from its stem. The current study is an attempt to delineate the milk clotting potential associated with its fruit extract and casein subunit specificity of the enzyme extract. The study also addresses the utility of phase partitioning (three phase partitioning) to concentrate the enzyme activity.

Cheese preparation and its Biochemistry

Earliest evidence of cheese making dated back to Neolithic age or 6th millennium BC. According to the legend, cheese was accidently discovered by an Arab merchant when he carried milk in bag made from skin of sheep's stomach around 8000 BC [9]. Detailed accounts of cheese manufacture, its characteristics and its culinary use in Roman history have been shown by researchers [10]. Cheese manufacturing and its use rose with spread of civilization. Monasteries and feudal estates played a major role in development of method of cheese making. Skills acquired through years of cheese making were passed on to the next generation that laid the foundation for cheese making through which varieties of cheese were developed using milk as the raw material [11]. This ancient milk derived product, rich in essential nutrients such as proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins and minerals is produced in various tastes, textures and shapes developed in different regions, climates and cultures of the world, valued for its portability and long shelf life.

Wrightia tinctoria and its utility in milk clotting

Wrightia tinctoria (Pala indigo plant or dyer's oleander) is a small deciduous tree which belongs to the family Apocynaceae [12]. The plant is distributed across Asia, Australia, Myanmar and Nepal. It grows up to 10 meters with milky latex, scaly, smooth and ivory coloured bark [13]. Also White, fragrant flowers are produced at the tip of the branches. Flowering and fruiting occurs between March and November [12]. Various parts of W.tinctoria including leaves, bark, and seeds exhibit medicinal properties and are being used in preparation of traditional medicines (Khyade 2014). Along with medicinal properties, it is also known to exhibit milk clotting activity. People of Kolli hills region of India use this property of this plant to prepare a local variety of cheese. 20 CE (crude enzyme) and TPP purified IP

(interracial phase) fractions of W.tinctoria stem exhibited milk clotting activity [14]. The raw latex and crude enzyme extract from W.tinctoria fruits have shown milk clotting activity against buffalo and goat milk, of which crude enzyme exhibited higher MCA (Jeganathan 2018).

MATERIALS AND METHODS

Plant source:

Authenticated *Wrightia tinctoria* (Authentication no: 64) fruit was collected from Indian institute of Horticultural Research (ICAR), Bangalore in the morning and was processed on the same day.

Scientific Classification	
Kingdom	Plantae
Order	Gentianales
Family	Apocynaceae
Genus	*Wrightia*
Species	***W. tinctoria***
Binomial name	*Wrightia tinctoria* (Roxb.) R.Br.
Vernacular Names	Dudhi, Beppale, Kodamurki, Paalai
Common Names	Dyer's Oleander, Pala Indigo, Sweet indrajao

Figure 1: Wrightia tinctoria unripe fruit [8]

Chemicals

Skimmed milk powder, ammonium per sulphate, t-butanol, trichloro acetic acid, casein, kappa-casein, disodium hydrogen phosphate, citric acid, tricine, acrylamide, bis- acrylamide, calcium chloride, bovine serum albumin, dialysis membrane, tri sodium citrate and tris base were procured from HiMedia laboratories Pvt.Ltd, Mumbai, India. Ethylene diamine tetra acetic acid (EDTA), Sodium dodecyl sulphate, Ammonium sulphate, Barium chloride, Folin-Ciocalteu reagent, sodium carbonate, tricine, TEMED, glycerol, glacial acetic acid, bromophenol blue, beta mercaptoethanol, Molecular marker (Bangalore Genei 14.3 to 97.4 kDa). Kappa casein was purchased from Sigma Aldrich. All chemicals procured were of analytical grade.

Crude enzyme preparation

The fruit was washed under flowing tap water followed by distilled water and was chopped, made into pieces and weighed. Briefly, 1g of the fruit was homogenised in 5 ml of distilled water using the pestle and mortar in chilled condition. The homogenised mixture was filtered using muslin cloth, and was centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was treated as crude enzyme (CE) and was stored at -20 °C till further analysis.

Three phase partitioning (TPP)

In a test tube, 5 ml of CE, required amounts of ammonium sulphate (40%, 60% and 80%) [1] was taken and 1:1 ratio of *t*-butanol was added and gently vortexed for few seconds and this was allowed to stand at RT for 1 hour to enable three phase separations into upper butanol phase, middle interfacial phase (IP) and lower aqueous phase (AP). The upper phase was discarded using Pasteur pipette and IP and AP were carefully removed separately. IP was dissolved in suitable amount of distilled water at 4°C. IP and AP fractions were then subjected to dialysis. Dialysis was carried out to remove the excess salt. It was carried out for four times with intervals of 1 hour in 1 litre distilled water in cold room. In between, the distilled water was checked for salt content using 10% barium chloride solution. After dialysis, the volume was noted down and the samples were preserved in -20°C till further analysis.

Protein Estimation

Protein content of CE, IPs and APs were determined by Lowry's method using bovine serum albumin (BSA) as the standard [2]. Different volumes of crude enzyme and TPP fractions were taken and the volume was made up to 1ml. 5 ml of alkaline copper solution was added, mixed thoroughly and allowed to stand at room temperature (RT) for 15 minutes. 0.6ml of dilute (1:1) Folin's reagent was added and incubated in dark for 30 minutes and absorbance at 660 nm was read.

Milk clotting activity (MCA)

Milk clotting activity (MCA) was assessed as per Arima et al., 1970. 1% skimmed milk powder was dissolved in 0.05M calcium chloride solution. 2 ml of substrate was incubated at 37°C for 5 minutes followed by addition of 200µl of the enzyme. The tubes were rotated at regular intervals and time taken for micelles formation was recorded.[3]

All the 7 fractions (Crude, IPs and APs) were assessed for their milk clotting potential. MCA was calculated following the formula below:

$$MCA\left(\frac{U}{ml}\right) = \frac{[2400\ X\ volume\ of\ substrate\ (ml)]}{[Time\ taken\ (\text{sec}) x\ volume\ of\ enzyme]}$$

Caseinolytic activity (CA)

CA of the CE and IP fractions were estimated according to Ladd and Butler, 1972 using tyrosine as standard (Ladd and Butler 1972: 19-30). Briefly, 1% of casein was prepared in 0.05M citrate phosphate buffer (pH 7.5). The solution was heat treated at 100° C for 15 minutes in a water bath, cooled and used as substrate. The reaction mixture consisted of 1ml of 1% casein, 200µl of CE/TPP fractions and 800µl of Citrate phosphate buffer. This reaction mixture was now incubated for one hour at room temperature and the reaction was terminated by adding 3 ml of 10% cold trichloro acetic acid (TCA). The tubes were allowed to stand for 1 hour at 2°C in ice to allow the undigested protein to precipitate. The mixtures were centrifuged at 6000 rpm for 10 minutes in RT. Absorbance of clear supernatant was measured at 280nm. CA was calculated using the following formula[4]:

$$CA\left(\frac{U}{ml}\right) = Tyr\ (\mu g) x \left(\frac{Dilution\ factor}{Vol.of\ enzyme\ (ml)}\right) x \left(\frac{Total\ volume\ (ml)}{Incubation\ time\ (min)}\right)$$

Milk Clotting Index (MCI)

Ratio of milk clotting activity to caseinolytic activity was calculated as milk clotting index (MCI)[5].

MCI = MCA / CA

Whole casein and kappa casein substrate specificity analysis through tricine SDS-PAGE

CE and IP fraction mediated whole and kappa casein hydrolytic pattern was evaluated using tricine SDS-PAGE.

Sample preparation

1%whole casein and 0.25% kappa casein were treated with CE and IP fractions. This was incubated for 1hr at room temperature. The reaction was stopped using 300µl of 10% chilled TCA. Then, this was centrifuged at 6000 rpm for 10 minutes at room temperature. The supernatant was discarded and pellet was dissolved in 1x sample buffer (4%SDS, 0.125M tris HCl, 10% mercaptoethanol, 0.4% Bromophenol blue, 20% glycerol).This, was heated in boiling water bath for 10 minutes and was allowed to cool and vortexed for few seconds and then loaded to respective wells marked earlier in the gel. Molecular marker (Bangalore Genei 14.3 to 97.4 kDa) was also prepared using the sample buffer as per the manufacturer's instructions. Negative control was prepared using whole casein and kappa casein without enzyme.

Gel preparation

The gel composition followed for stacking gel was 4%T&3%C and for separating gel was 16.5% T & 3% C [6].

Top reservoir was filled with cathode buffer and the bottom reservoir was filled with anode buffer. Control, sample and marker was loaded to the respective wells (20µl) each. Electrophoresis was performed at room temperature using 50V until the tracking dye just crossed the stacking gel and then the voltage was increased to 100V till the dye reaches the bottom of the gel. The gel was then fixed for 30 minutes in the fixer containing 10% glacial acetic acid, 50% methanol and 40ml of distilled water. Then the gels were stained overnight with 0.025% of Coomassie Brilliant Blue (R250). Subsequently, the gel was destained 3-4times using 10% glacial acetic acid solution and the gel was visually analysed followed by documentation.

Statistical analysis

The data obtained from five independent experiments were analysed using Graph Pad Prism (9.1.0). Data are expressed as mean ± SD. Each value represents the mean of five independent experiments performed in triplicates. Data were subjected to one way ANOVA to find significant difference at 5% significance level in the mean values wherever applicable and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Protein estimation by Lowry's method

Analysis of the mean values of protein concentration between the IP and AP fractions revealed that AP fractions had more protein when compared to IP fractions (Table2).

Table 2: Protein concentration of crude extract and TPP fractions of W. tinctoria

***W.tinctoria* fruit extract**	**Protein concentration (mg/ml)**			**Mean protein concentration (mg/ml)**	**±SD**
	Trial 1	**Trial 2**	**Trial 3**		
Crude	1.2	1.2	1.13	1.18	0.04
IP40	0.3	0.3	0.3	0.3	0
IP60	0.2	0.18	0.2	0.19	0.01
IP80	0.2	0.18	0.18	0.18	0.01
AP40	0.51	0.51	0.53	0.51	0.01
AP60	0.43	0.4	0.4	0.41	0.01
AP80	0.3	0.33	0.3	0.31	0.01

MCA of crude extract and TPP fractions

MCA of the CE gave a mean value of 1764. Compared to CE, IP40 gave a lower MCA. However, IP60 was 1.2 times greater than CE and IP80 was approximately 1.3 times greater than the CE (Table 3). All the AP fractions (AP40, AP60, and AP80) exhibited very low MCA when compared to crude and IP fractions. When the assay was extended to check the time taken by each of these enzyme source to induce a solid milk clot, it was found that the CE took approximately 20 minutes and IP60 and IP80 took approximately 26-27 minutes. IP40 and AP40 took over 1 hour for the total solidification of the milk. AP60 and AP80 did not contribute to any clotting even after 2 hours (Table 4).

Considering their higher MCA and lesser time they took for inducing the solid milk clot formation, IP60 and IP80 have been taken forward to further detailed analysis. IP40 and all AP fractions have not been considered further. Table 3 is a compilation of the MCA by crude and TPP fractions. The Fig.5.1 is a representative image of the milk clotting performance by crude extract and TPP fractions (IP60, IP80) of *W.tinctoria.*

Table 3: MCA of crude extract and TPP fractions of *W. tinctoria*

W.tinctoria fruit extract	Milk Clotting activity (MCA Units)			Mean MCA Units	±SD
	Trial 1	Trial 2	Trial 3		
Crude	1846.15	1846.15	1600	1764.1	142.11
IP40	1600	1600	1600	1600	0
IP60	2181.81	2181.81	2000	2121.20	104.96
IP80	2400	2400	2181.81	2327.27	125.97
AP40	282.3	282.3	266.6	277.06	9.06
AP60	750	750	436.3	645.43	181.11
AP80	342.8	342.8	333.3	339.63	5.48

Table 4: Time taken by the enzyme extracts for solid milk clot formation

W.tinctoria fruit extract	Time in minutes			Mean (Min)	±SD
	Trial 1	Trial 2	Trial 3		
Crude	20	20	22	20.66	1.15
IP40	60	62	63	61.66	1.52
IP60	25	27	28	26.66	1.52
IP80	25	28	29	27.33	2.08
AP40	70	70	72	70.66	1.15
AP60	No clot formed till 2 hours	No clot formed till 2 hours	No clot formed till 2 hours	-	-
AP80	No clot formed till 2 hours	No clot formed till 2 hours	No clot formed till 2 hours	-	-

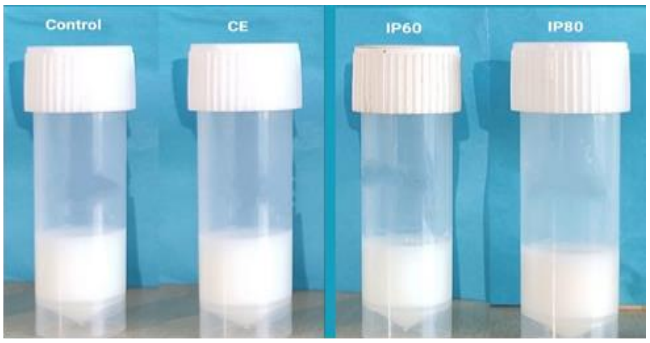

Figure 2. MCA of crude extract and TPP fractions of *W. tinctoria*

Caseinolytic activity of crude extract, IP60, IP80 fractions

CE exhibited a very high CA compared to both IP60 and IP80 fractions (Table 5). Concentration of these proteases through TPP has brought their CA down to 6 times decrease in IP 60 (p value 0.0007) and 4.5 times decrease in IP 80 (p value 0.0007)compared to CE. It is interesting to note that even with low CA; their MCA was higher when compared to the CE. This clearly suggested the effectiveness of three phase partitioning to facilitate the concentration of those proteases that are contributing specifically to milk clotting.

Table 5: CA of *W. tinctoria* fruit extracts

W.tinctoria fruit extract	Caseinolytic activity (CA units)			Mean CA units	±SD
	Trial 1	Trial 2	Trial 3		
Crude	68.92	63.85	63.85	65.54	2.92
IP60	10.97	10.99	10.55	10.83	0.24
IP 80	16.44	13.45	13.45	14.44	1.72

Milk clotting index of crude extract, IP60, IP80

Comparison of MCI values between CE and IP fractions reveals three phase partitioning has contributed to enhancement of MCI to 7 times in IP60 (p value 0.0033) and approximately 6 times (p value 0.0033) for 1P80, when compared to the CE (Table 6).

Table 6: Milk Clotting In dex of CE and IPs

W.tinctoria fruit extract	MCI (MCA/CA)			Mean MCI	±SD
	Trial 1	Trial 2	Trial 3		
Crude	26.78	28.91	25.05	26.91	1.93
IP60	198.88	198.52	189.57	195.65	5.27
IP 80	145.98	178.43	165.91	163.44	16.36

NATURE OF WHOLE CASEIN AND KAPPA CASEIN HYDROLYSIS BY CE, IP60 AND IP80.

Incubation of whole casein with CE, IP60 andIP80 resulted in complete hydrolysis of all subunits of casein, i.e. αs2, αs1, β and κ caseins. The control casein presented (incomplete separation) two prominent bands between 20 and 29 kDa positions in the gel. The lanes of CE, IP60 and IP80 exhibited complete disappearance of these bands and presence of a wide band almost like a smear at a lower molecular weight position in the gel. However in the lanes of IP60 and IP80, two protein bands at around 14.3 kDa position were clearer (Fig.5.2)

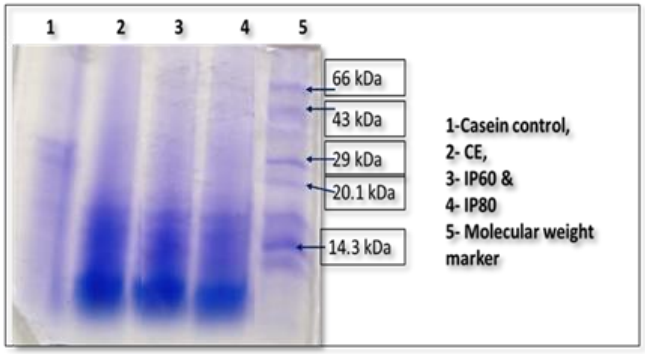

Figure 3: Whole Casein Hydrolysis by CE, IP 60 and IP80

Treatment of these enzymes with kappa casein alone showed disappearance of the kappa-casein band that was present in the control lane of the gel. The lane where the crude enzyme treated kappa-casein was loaded; a smear of stained area was obtained. Whereas, the lanes where the IP60 and IP80 treated kappa-casein were loaded showed the presence of 2-3 prominent lower molecular weight bands (Fig. 5.3). These bands appear to be at the same position to that of two protein bands at around 14.3 kDa position seen in the whole casein hydrolysis pattern. The results suggest TPP may have favoured separation and concentration of proteases with certain bond specificity on kappa casein subunit and justify the reason behind the higher MCA and lower CA of IP 60 and IP 80 compared to CE.

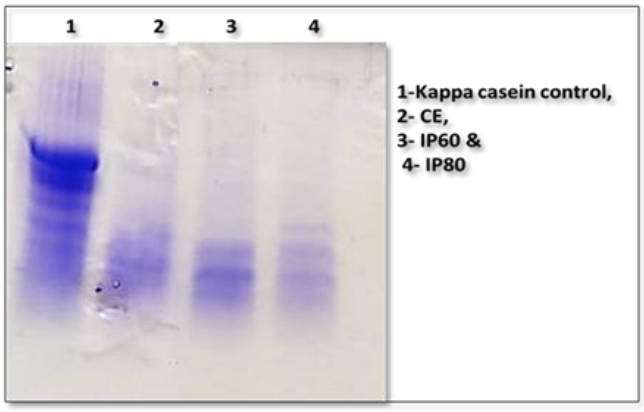

Figure 4: κ Casein Hydrolysis by CE, IP 60 and IP80

CONCLUSION

Based on the results obtained in the present study, it can be concluded that crude extract from *W.tinctoria* fruit can coagulate milk. Milk clotting proteases in the CE had more specific affinity towards κ-casein compared to other subunits of casein. Study also provides evidence towards the utility of TPP in concentrating these milk clotting proteases and in improving their MCI.

FUTURE DIRECTIONS

The study can be taken forward to:

- Examine the influence of different salt concentration and ratio of enzyme extract tot-butanol for its maximum recovery and improved substrate interaction
- Prepare cheese using purified enzyme and study its nutritional characteristics
- Understand immobilisation prospects of these proteases through suitable system for making enzymic milk coagulation economically feasible
- Study the utility of these proteases in the development of bio active peptide/functional food

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Declaration of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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