ISSN PRINT 2319 1775 Online 2320 7876
Research Paper **© 2012 IJFANS. All Rights Reserved** UGC CARE Listed (Group -I) Journal Volume 10, Iss 02, 2021

BIOCHEMICAL STUDIES OF COCOONASE FROM FOUR BREEDS OF MULBERRY SILKWORM BOMBYX MORI L.

P. H.Thejaswini[1]*, H.B.Mahesha[2], B.A. Parthasarathi[3]

[1]Department of Biotechnology, Maharani's Science College for Women, Mysuru-570 005.
[2]Department of Sericulture, Yuvaraja's College, University of Mysore, Mysuru-570 005,
[3]Department of Sericulture, Manasagangotri, University of Mysore, Mysuru-570 006. India.

***Corresponding Author:** P. H.Thejaswini
thejaswiniph@gmail.com

ABSTRACT

Cocoonase collected from two multivoltine breeds of mulberry silkworm namely Nistari, C. nichi, and two bivoltine breeds namely KA and NB_4D_2. The crude enzyme was isolated and purified by column chromatography. The bivoltines yielded 750 mgs, where as multivoltines yielded 670 mgs for every gram of crude sample loaded on to DEAE-Cellulose column. The analysis of electrophoretic mobility of bound protein from DEAE-Cellulose column and that of Fraction-II of sephadex gel filtration displayed similarity in molecular weight. The results of amino acid analysis exhibited the results that the amount of aromatic amino acids is very less and tryptophane is found to be completely absent. The results of paper chromatography of carbohydrates showed presence of glucose like substance in the cocoonase sample of all the breeds.

Key Words: Chromatography, Gel Filtration, Electrophoresis, Amino acid analysis.

1. INTRODUCTION

Cocoonases are a group of proteases produced by silk moths for the purpose of softening the end of cocoon so as to permit the moth to escape from its shell [1], [2]. As the pupa develops into an adult, epidermal cells in the maxillary galea secrete a concentrated solution of a homogeneous proteolytic enzyme. The droplets get dried up into partial crystalline form on the galea, which may be harvested easily with fine forceps in case of Antheraea mylitta, A. pernyi and A. polyphemus. In contrast, because of its smaller size and lesser secretion it is very difficult to harvest the enzyme from Bombyx mori. In natural condition, the moth secretes a buffer which dissolves the solid enzyme for the deployment. Each galea may produce nearly 0.1mg of enzyme [3].

Under a variety of conditions of electrophoretic analyses the crude cocoonase has been shown to migrate as a single cationic band. This was proved by starch gel electrophoresis (pH 8.6), acrylamide gel electrophoresis (pH 4.0), agar coated slides (pH 7.0) and Sepharose-III (pH 8.3 and 9.5). When cocoonase from A. pernyi and A. polyphemus was analyzed using cellulose acetate with 0.5M Tris-HCl (pH 8.3), a faint band trailing behind the major band was reported [4].

Sodium Dodecyl Sulphate Poly acrylamide Gel Electrophoresis (SDS-PAGSE) is proved to be a useful tool for the separation of protein sub units and the determination of their molecular weight. The molecular weight of the cocoonase enzyme from A. mylitta and A. polyphemus was estimated by SDS-PAGE to be 25,000 $\pm$ 500 and 28400 $\pm$ 400 Dalton

Research Paper **© 2012 IJFANS. All Rights Reserved**

respectively [5]. The gel filtration can also be used to estimate the molecular weight of proteins [6]).

The cocoonase enzyme on gel filtration by Sephadex G-75 showed in addition to the major peak, a minor component devoid of esterase activity, but on Sephadex G-100 gave a single peak of enzymatically active material [4]. The molecular weight of A. mylitta and A. polyphemus was estimated by Sephadex G-100 column chromatography and was found to be 22,100 $\pm$ 1000 and 24,700 $\pm$ 1000 respectively [5], which is comparable with the value obtained by Hruska and Law [7] and Felsted et al., [8].

In ion exchange chromatography at pH 8, prococoonase behaved as an anion, binding to DEAE-Cellulose but not to CM-Cellulose. By contrast, active cocoonase behaved as cation binding to CM-Cellulose but not to DEAE-Cellulose [2], [4], [9].

Quantitative determination of various amino acids in an enzyme bears a direct relationship to the chemistry of proteins. Amino acid analysis provides evidence for purity of a protein, and it is one of the first steps in the elucidation of chemical structure of a protein molecule.

The amino acid composition of cocoonase is generally similar to that of mammalian trypsin and chymotrypsin. But, an exception is that the cysteine content is either 4 or 6 residues per molecule for cocoonase but 12 for bovine trypsin. Contrary to early indication [2], [4] all cysteine of cocoonases are involved in the formation of disulphide bridges [10]. Thus, the presence of hardly a few disulphide bridges is responsible for the instability of cocoonase at the extremes of temperature and pH, and also in the presence of urea [2], [4].

The cocoonase enzymes from A. polyphemus and A. pernyi are reported to contain carbohydrate moieties [10]. When they were qualitatively analyzed, both the enzymes were found to contain glucosamine and mannose. In contrast, the cocoonase of A. mylitta was found to lack carbohydrate [10]. However, biochemical studies on the cocoonase from mulberry silkworm Bombyx mori L., are rather scarce. Hence, the present investigation was undertaken.

2. MATERIALS AND METHODS

Four breeds mulberry silkworms belonging to multivoltine (C. niche & Nistari) and bivoltine (Kalimpong-A & NB_4D_2) were reared in the laboratory following the method described by Krishnaswami [11].

On the eighth day of pupation, the cocoons were cut open and the naked pupae were preserved at room temperature. The moths emerge on the tenth day from the pupal case; secrete the buffer, which dissolves the enzyme that was already deposited on galea (mouth part) in a crystalline form. These secretions were collected in the test tubes as shown in the Fig. 1 [12]. Such cocoonase was preserved at -15°C in the deep freezer for further use.

Research Paper **© 2012 IJFANS. All Rights Reserved**



Fig. 1 Collection of Cocoonase

The total protein present in crude cocoonase was determined by following the method of Lowry et al., [13]. Poly acrylamide gel electrophoresis (PAGE) was carried out according to Davis [14].

Column chromatography was carried out as follows. Carboxymethyl Cellulose (CM-Cellulose) and diethyl amino ethyl cellulose (DEAE-Cellulose) were processed as specified by the Sigma chemicals. The cellulose matrix was packed into a glass column of 1.5 x 5 cm and equilibrated with 0.01 M phosphate buffer pH 7. After loading the sample the column was eluted by gradient of phosphate buffer containing KCl (0.05-0.2M). Fractions of 2 ml each were collected using automatic fraction collector (LKB) at a flow rate of 30 ml/h and their absorbance at 280 nm was recorded using Hitachi Spectrophotometer.

Sephadex G-100 was used for gel filtration. The swelled Sephadex G-100 was packed into a 2 x 40 cm glass column, and it was equilibrated with 0.01M phosphate buffer, pH 7. The loading and elution of samples were carried out at 4°C. Fractions of 2.0 ml each were collected at a flow rate of 15 ml/h with the help of automatic fraction collector. Each fraction was monitored at 280 nm using Hitachi Spectrophotometer. Fractions containing more protein were pooled, dialyzed and freeze dried.

Molecular weight of cocoonases was determined using both Sephadex G-100 column chromatography [6] and SDS-PAGE. Relative mobility of the marker proteins and the enzyme in SDS-PAGE were calculated as described by Weber and Osborn [15]. A plot of relative mobilities (Rf) of the marker proteins versus molecular weight was used to determine the molecular weight of the cocoonase.

Determination of amino acid composition was done according to the procedure of Moore and Stein [16]. Concentration of amino acid in the sample was quantified by comparison of the

Research Paper **© 2012 IJFANS. All Rights Reserved**

amount per area of amino acid with that of the standard obtained by recordings of LKB integrator (Broma).

Estimation of sugar was carried out by phenol-sulphuric acid method [12]. Detection of sugar by chromatography was carried out as follows. The hydrolyzed samples of cocoonase and markers were applied on What man No. 1 filter paper at equal distances in the following order. i. Glucose, ii. Mannose, iii. Galactose, iv. Xylose, v. Ribose, vi. Arabinose, vii. Rhamnose, viii. Glucuronic acid, ix. Acetyl glucosamine and samples a. NB_4D_2, b. KA, c. Nistari and d. C. nichi. The spots were dried during application by blowing hot air. When the solvent mixture of Butanol:Acetic acid:Water in the ratio of 4:1:1 (v/v/v) almost reached the other edge of the paper, it was removed and dried in air. After dissolving 0.8 gm of oxalic acid in 50 ml of distilled water, 0.5 ml of aniline was added and shaken well. This aniline-oxalate reagent was sprayed uniformly on the paper and dried in an oven at 199-110 C for 3-5 minutes or until the spots show up on the paper. The sample was compared with those of the standard sugars.

3. RESULTS AND DISCUSSION

The crude cocoonase from all the four breeds of Bombyx mori was used for isolation and purification of active factor in the enzyme. Ion-exchange chromatography using DEAE-Cellulose and CM-Cellulose was carried out for crude cocoonase from KA, NB_4D_2, Nistari and C. nichi breeds of Bombyx mori. The chromatograms of individual breed from DEAE-Cellulose column are given in Fig. 2-5. The crude samples of all the breeds did not bind to CM-Cellulose, in which the quantity of unbound material was found to be more or less similar to that loaded onto the column.

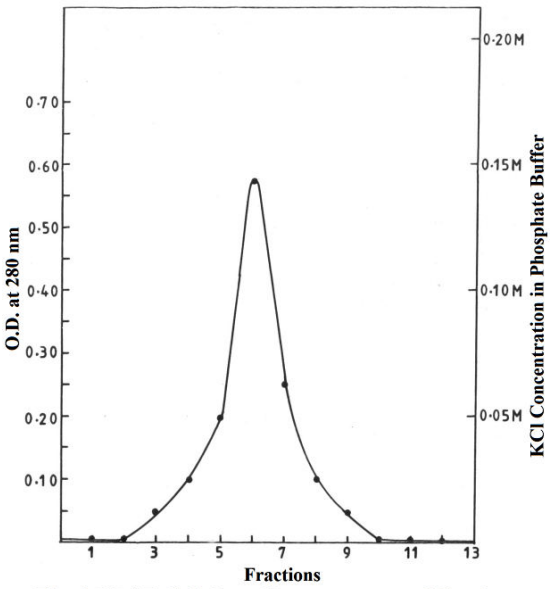


Fig. 2 DEAE-Cellulose Chromatogram of Crude Cocoonase from KA

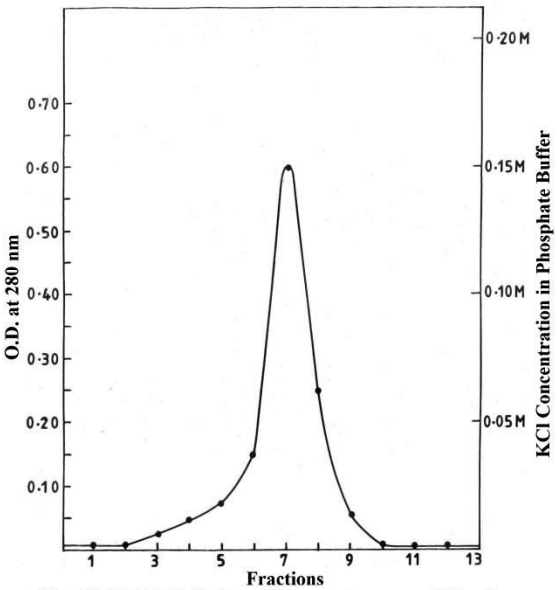


Fig. 3 DEAE-Cellulose Chromatogram of Crude Cocoonase from NB4D2

Research Paper **© 2012 IJFANS. All Rights Reserved**

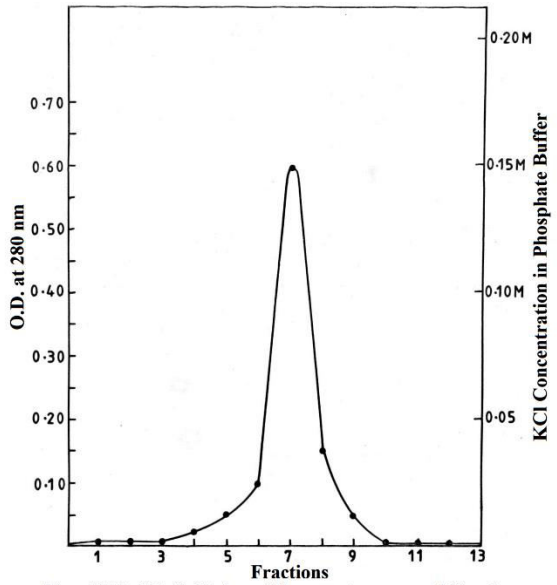


Fig. 4 DEAE-Cellulose Chromatogram of Crude Cocoonase from Nistari

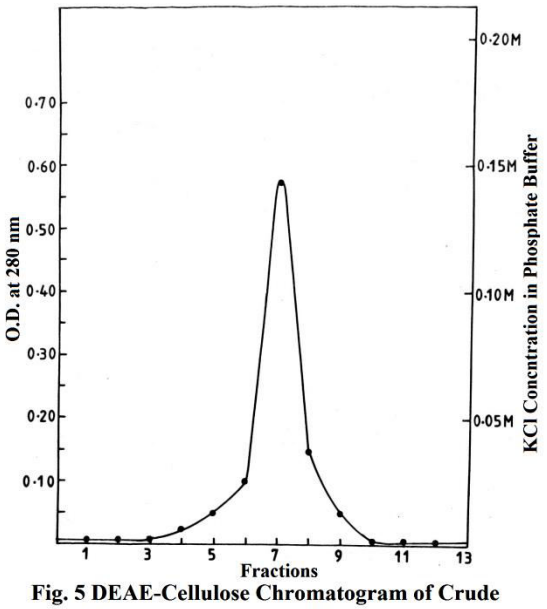


Fig. 5 DEAE-Cellulose Chromatogram of Crude Cocoonase from C. nichi

On the contrary, in DEAE-Cellulose column, unbound material showed lesser protein as compared to that loaded onto the column. Hence, by increasing the salt (KCl) concentration in the eluting buffer more amount of protein as compared to unbound protein was eluted out as represented in Fig. 2-5. The amount of bound protein in bivoltine breeds was found to be about 750 mgs, where as in multivoltines about 670 mgs (Table 1).

TABLE 1. AMOUNT OF PROTEIN IN CRUDE SAMPLE

Sl. No.	Breed	Amount of unbound proteins (mgs)	Amount of bound Proteins (mgs)
1	KA	202	755
2	NB_4D_2	240	742
3	Nistari	228	672
4	C. nichi	289	669

Note: Amount of protein loaded on to DEAE-column was 1.0g.

Sephadex G-100 gel filtration showed two peaks from the crude samples of cocoonase from each breed as shown in Fig. 6-9.

ISSN PRINT 2319 1775 Online 2320 7876
Research Paper **© 2012 IJFANS. All Rights Reserved** UGC CARE Listed (Group -I) Journal Volume 10, Iss 02, 2021

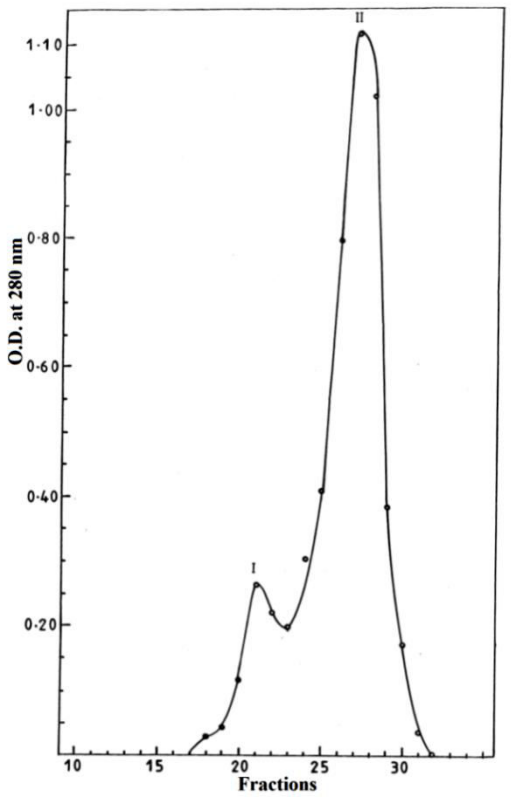


Fig. 6 Elution Profile of Gel Filtration using crude Cocoonase from KA. I=Fraction I; II= Fracton II.

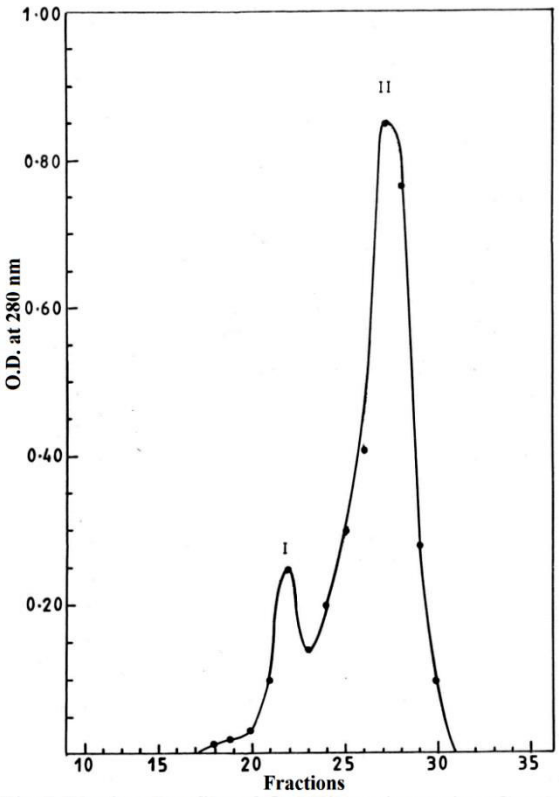


Fig.7 Elution Profile of Gel Filtration using Crude Cocoonase fron NB4D2. I=Fraction I; II=Fraction II.

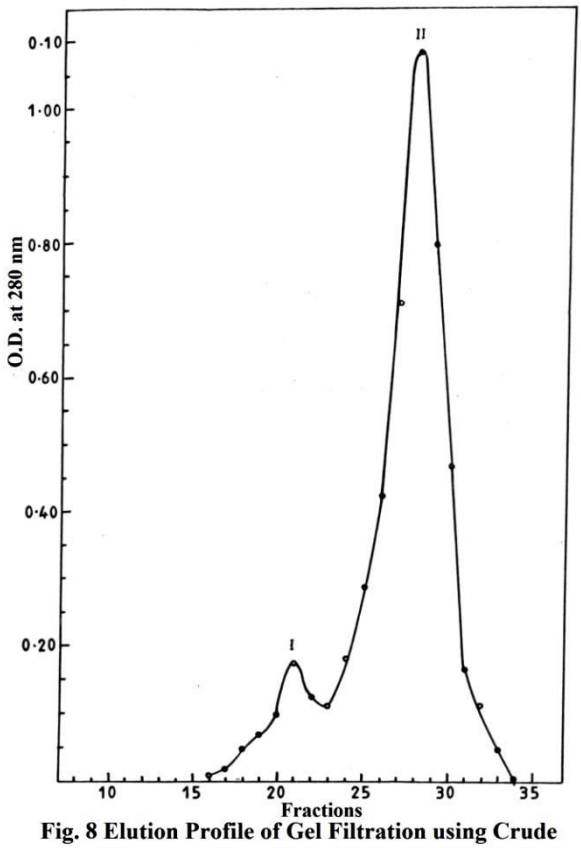


Fig. 8 Elution Profile of Gel Filtration using Crude Cocoonase fron Nistari. I=Fraction I; II=Fraction II.

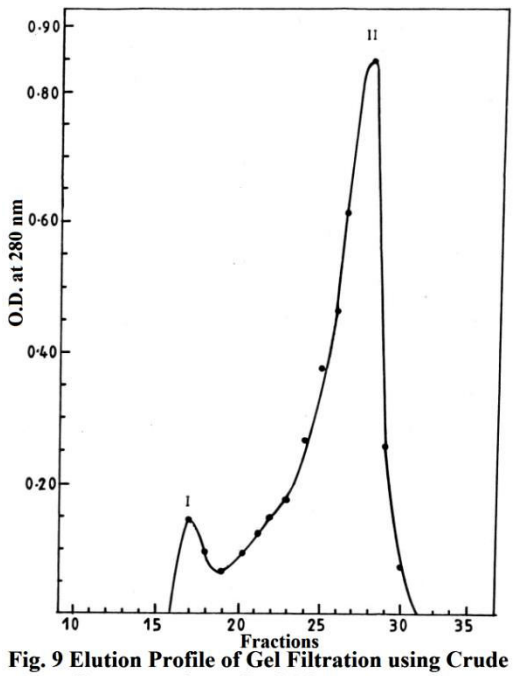


Fig. 9 Elution Profile of Gel Filtration using Crude Cocoonase from C. nichi. I=Fraction I; II=Fraction II.

Research Paper **© 2012 IJFANS. All Rights Reserved**

The smaller peak eluted in the void volume is found to have significantly lesser protein as compared to that of larger peak (Table 2).

TABLE 2. AMOUNT OF PROTEIN IN DIFFERENT FRACTIONS

Sl. No.	Breed	Protein in Fraction-I (mgs)	Protein in Fraction-II (mgs)
1	KA	204	789
2	NB_4D_2	240	702
3	Nistari	205	270
4	C. nichi	210	705

Note: Amount of protein loaded on to Sephadex G-100 column was 1.0g.

The first (smaller) peak probably represents the unbound protein and the second (larger) peak might correspond to that of bound protein obtained in DEAE-Cellulose column. The filtration pattern of the marker protein like ovalbumin, pepsin, trypsinogen, myoglobin and cytochrome-C, each of different molecular weight was used to estimate the molecular weight of cocoonase of different breeds by following the procedure of Andrews (1965). The calibration curve for the estimation of molecular weight is given in Fig. 10.

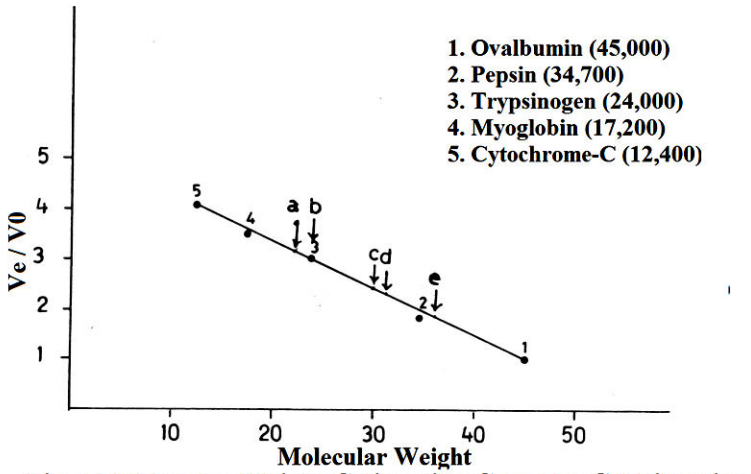


Fig. 10 Molecular Weight Calibration Curve by Gel Filtration
a. Fraction I from Nistari and C. nichi; b. Fraction II from KA and NB4D2
c. Fraction I from NB4D2; d. Fraction I from KA and Nistari
e. Fraction I from C. nichi

The molecular weight determined based on the filtration pattern of Fraction-II of cocoonase from bivoltine breeds like NB_4D_2 and KA show 24,000 Daltons and that of multivoltine breeds like Nistari and C. nichi show 23,000 Daltons. The Fraction-I showed no uniformity in molecular weight when compared between different breeds (Table 3).

TABLE 3. MOLECULAR WEIGHT BY GEL FILTRATION (ANDREW'S METHOD)

Sl. No.	Breed	Fraction-I (Daltons)	Fraction-II (Daltons)
1	KA	30,000	24,000
2	NB_4D_2	31,000	24,000

Research Paper **© 2012 IJFANS. All Rights Reserved**

3	Nistari	31,000	23,000
4	C. nichi	36,000	23,000

The crude cocoonase samples from all the four breeds were analyzed by PAGE. All the breeds showed similar zymogram pattern with two bands as in Fig. 11A. The electrophoretic analysis of the protein bound to DEAE-Cellulose column (Fig. 12A) showed a single band. Similar results were also obtained on the analysis Fraction-I (Fig. 11B) and Fraction-II (Fig. 11C) obtained by gel filtration. Although each showed single band, the Fraction-I is comparable to the band of slow mobility, where as that of Fraction-II is comparable to the band of fast mobility of crude cocoonase. When Fraction-II was analyzed using (1%) SDS (Fig. 12B), it was found that the protein showed faster mobility as compared to its movement in PAGE without SDS.

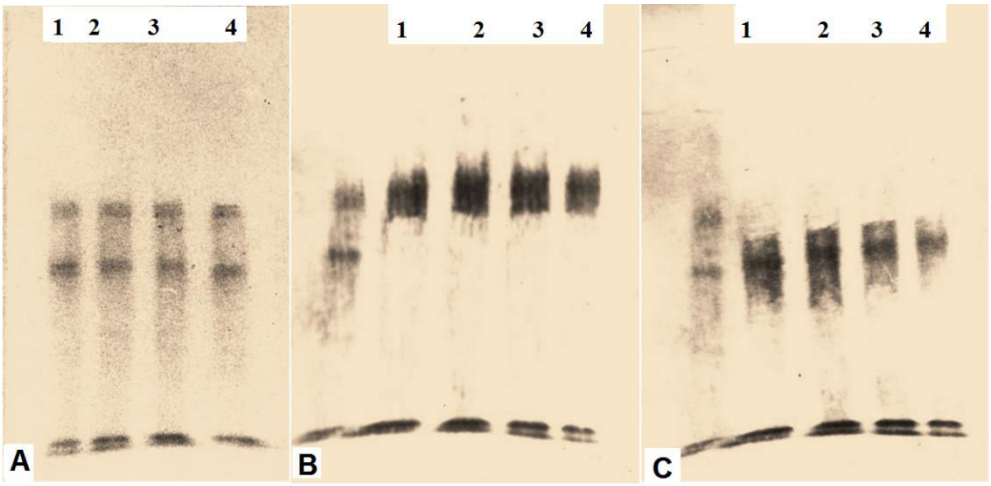


Fig. 11 Analysis by PAGE

A-Crude Cocoonase; B-Fraction I from Sephadex G-100 Gel Filtration
C-Fraction II from Sephadex G-100 Gel Filtration
Lane-1:KA; Lane 2: NB4D2; Lane 3: Nistari; Lane 4: C. nichi

Research Paper **© 2012 IJFANS. All Rights Reserved**

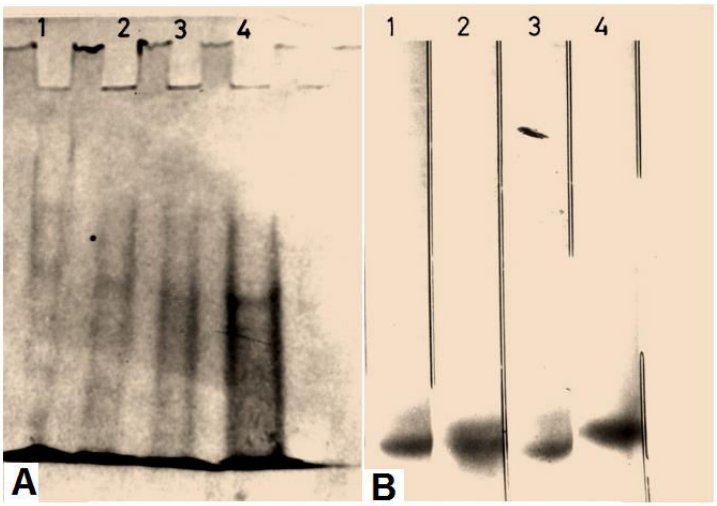


Fig. 12 Analysis by PAGE
A-Fraction bound to DEAE-Cellulose
B-Fraction II from gel filtration, using SDS-PAGE
Lane 1: KA; Lane 2:NB4D2; Lane 3: Nistari; Lane 4: C. nichi

The purified cocoonase (Fraction-II from Gel Filtration) of all the four breeds was analyzed by SDS-PAGE (Fig. 13) to determine molecular weight using different markers like pepsin, trypsinogen, lactoglobulin and lysozyme.

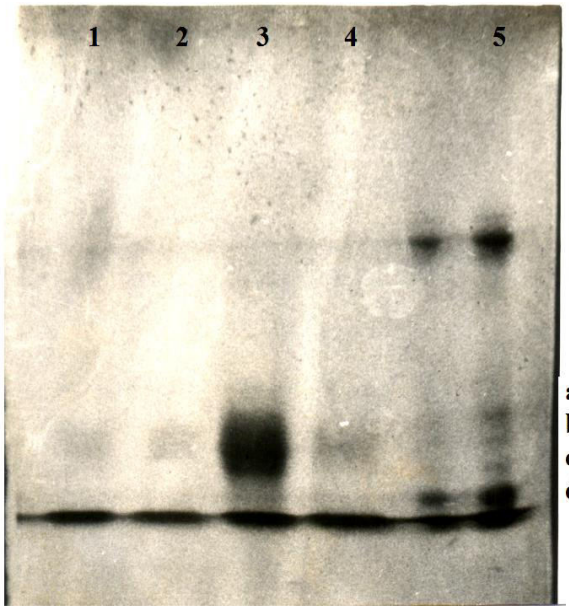


Fig. 13 Estimation of Molecular Weight of Fraction II by SDS-PAGE
Lane 1: KA; Lane 2: NB4D2; Lane 3: Nistari; Lane 4: C. nichi; Lane 5: Marker Proteins

A calibration curve of the markers is represented in Fig. 14. The cocoonase of all breeds seems to correspond with the molecular weight of trypsinogen (24,000 Daltons).

Research Paper **© 2012 IJFANS. All Rights Reserved**

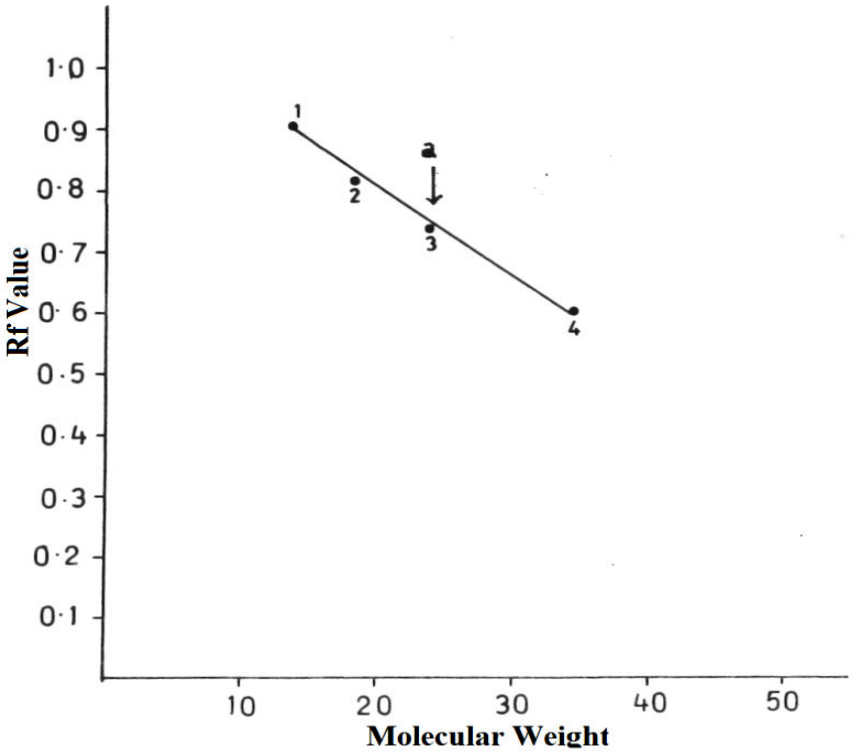


Fig. 14 Molecular Weight Calibration by SDS-PAGE
Note:'a' represents the position of Molecular Weight of Fraction II from all breeds

The qualitative analysis of carbohydrate in Fraction-II was carried out by paper chromatography (Fig. 15) using various markers.

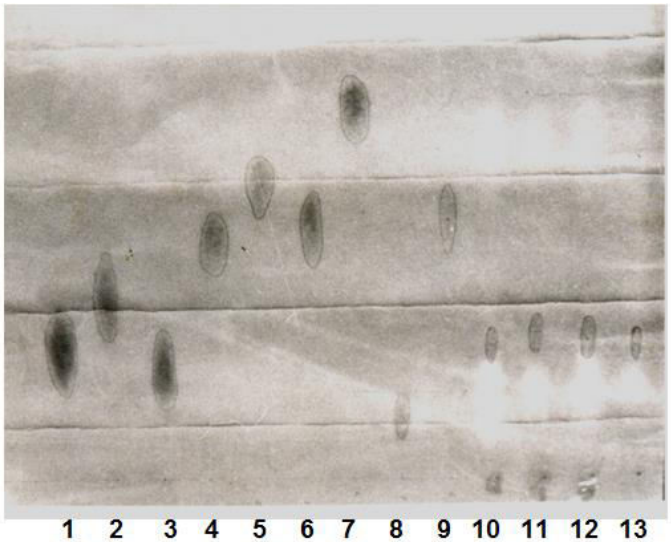


Fig. 15 Paper Chromatography of Fraction II
Lane 1: Glucose; Lane 2:Mannose; Lane 3: Galactose
Lane 4: Xylose Lane 5: Ribose; Lane 6: Arabinose
Lane 7: Rhamnose; Lane 8: Glucuronic acid
Lane 9. Acetyl glucosamine
Lane 10: NB4D2; Lane 11: KA; Lane 12: Nistari; Lane 13 C. nichi

The histogram based on the Rf value, is shown in Fig. 16. As evident in this figure, the Fraction-II of all the breeds appears to have similar type of carbohydrate.

Research Paper **© 2012 IJFANS. All Rights Reserved**

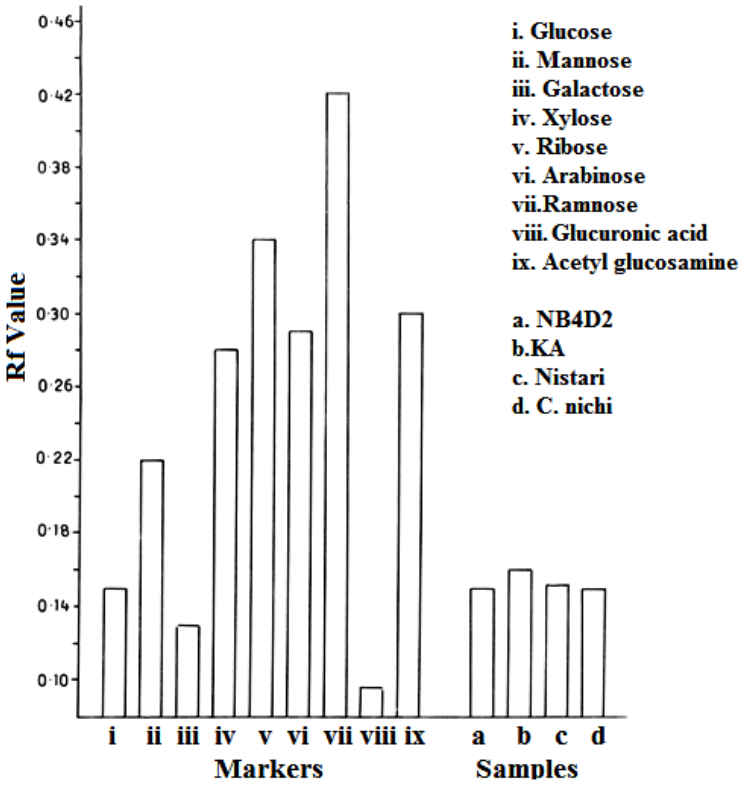


Fig. 16 Histogram of Paper Chromatography from Fraction II

The comparison of carbohydrate in purified cocoonase and crude enzyme reveals that each breed seems to have different amount of carbohydrate as shown in Table 4.

TABLE 4. QUANTITATIVE ANALYSIS OF CARBOHYDRATES

Sl. No.	Breed	Crude Sample (mg/g)	Purified Sample (mg/g)
1	KA	0.987	0.093
2	NB_4D_2	0.607	0.101
3	Nistari	0.818	0.069
4	C. nichi	0.866	0.079

The result of amino acid analysis cocoonase of each breed (Table 5) found to be variable in different breed. While of purified it has been some amino acids were found to be significantly more, certain amino acids exist in trace quantity, although tryptophane was found to be completely absent in all the breeds.

TABLE 5. AMINO ACID ANALYSIS OF COCOONASE Residues/Molecule of Protein

Sl. No.	Amino Acids	KA	NB4D2	C. nichi	Nistari
1	Glycine	0.06	0.73	1.33	2.28
2	Alanine	1.31	0.19	2.51	2.97
3	Valine	0.33	2.41	1.86	2.08
4	Leucine	0.85	0.14	0.01	0.38

Research Paper **© 2012 IJFANS. All Rights Reserved**

5	Isoleucine	0.83	0.05	0.91	0.35
6	Phenylalanine	1.47	0.51	Traces	1.25
7	Tryptophane	Nil	Nil	Nil	Nil
8	Tyrosine	0.72	Traces	Traces	1.38
9	Serine	0.01	0.18	2.02	1.79
10	Threonine	1.51	0.16	2.22	2.65
11	Aspartic acid	5.57	3.72	2.78	2.95
12	Glutamic acid	5.40	5.92	7.28	9.15
13	Lysine	1.78	0.57	1.30	0.83
14	Arginine	Traces	1.92	Traces	Traces
15	Histidine	Traces	Traces	2.18	Traces
16	Cysteine	Traces	Traces	Traces	Traces
17	Methionine	2.26	0.20	Traces	Traces
18	Proline	Traces	Traces	Traces	Traces
19	Ammonia	0.41	0.30	0.16	0.06

The failure of cocoonase to bind to cation exchanger like CM-Cellulose indicates that the crude sample appears to be rich in acidic amino acids. This is confirmed because cocoonases bind to DEAE-Cellulose (Fig. 1-4), the anion exchanger (Berger et al., 1971). The unbound protein collected in the void volume in DEAE-Cellulose column might also have acidic amino acids or more of basic amino acids but with least affinity towards DEAE-Cellulose. However, it needs further characterization. The bound protein showed the activity of true cocoonase. Hence, this fraction was used in all other studies.

The result of gel filtration (Fig. 5-8) reflects that the DEAE-Cellulose unbound protein represents Fraction-I and that which was bound as Fraction-II in the four breeds. In conformity with ion exchange chromatographic results, Fraction-I of gel filtration is found to be less and Fraction-II is more quantitatively. Thus, Fraction-II in gel filtration and the bound protein of ion exchange chromatography have been found to be similar in character. Hence, such purified fractions of DEAE-Cellulose, as well as Sephadex G-100 column have been characterized. Quantity wise, the first fraction is found to be negligible as compared to the active factor found in Fraction-II. Further, protein obtained in the first fraction by filtration method was found to be of high molecular weight as evidenced by zymogram of PAGE (Fig. 2B). The analysis of electrophoretic mobility of bound protein from DEAE-Cellulose column and that of Fraction-II from gel filtration indicates the similarity in molecular weight. And, it has been estimated to be around 24,000 Daltons, based on the relative mobility with the marker proteins. However, the purified cocoonase obtained by gel filtration method shows faster mobility in the SDS-PAGE as compared to that obtained without SDS. Such, difference of mobility in the absence or presence of SDS may be due to the break of disulphide bond between cysteine with consequent exposure of positively charged amino acids in the molecule of cocoonase. However, only single band of protein was found irrespective of SDS. Such a behavior of cocoonase in relation to SDS also suggests that the enzyme is a single polypeptide chain. By virtue of the comparability in the mobility of cocoonase and the markers, it has been found that purified cocoonase of all the breeds

Research Paper **© 2012 IJFANS. All Rights Reserved**

display higher molecular weight in contrast to that obtained in the zymogram without using SDS. Such a difference in molecular weight might be attributable to the relaxation of the complex molecule in the presence of SDS.

As shown in Table 6, the amount of enzymatic protein was found to be more in bivoltines and less in multivoltines corresponding to the pupal weight, shell weight and shell ratio of each breed. Such relationship of more protein in bivoltine and less in multivoltine in the crude sample reflected in the amount of protein obtained by ion exchange chromatography, and seems to have direct bearing on the texture of cocoon as well as the functional feature of cocoonase, as evidenced by Table 6. The enzyme in the crude sample seems to be active in alkaline pH (pH 8 approximately) regardless of the breeds.

The analysis of purified fraction of cocoonase from both bivoltines and multivoltines by paper chromatography (Fig. 15 and 16), indicates that the enzyme is a glycoprotein (Kramer et al., 1967). The comparison of carbohydrate in the purified fraction with that of markers indicates that the cocoonase enzyme seems to have glucose like carbohydrate. In addition, the relative concentration of carbohydrate in purified fraction and crude sample appears to be not uniform (Table 4), in the sense that the amount of carbohydrate does not show any correlation with specific breed, either bi or multivoltines.

The histogram obtained for the markers and the purified cocoonase shows that the glucose-like carbohydrate found to be more or less similar in all the breeds tested. The amino acid analysis of the purified cocoonase from four breeds of mulberry silkworm Bombyx mori was carried out according to the procedure of Moore and Stain (1963). The analysis shows that acidic amino acids are relatively more as compared to others in all the four breeds of Bombyx mori, particularly glutamic acid is more in all the breeds when compared to aspartic acid (Table 5). The amount of aspartic acid is more in bivoltines than in multivoltines, whereas, the amount of glutamic acid is more in multivoltines than in bivoltines. The aliphatic amino acids particularly glycine, alanine and valine quantity is more in multivoltines, specifically in Nistari, than among bivoltines. But the amount of leucine and isoleucine are more of less same in all the four breeds. On the whole, the amount of aromatic amino acids are very less, that too tryptophane is completely absent in all the breeds. Considerable amount of phenylalanine and tryosine is present in KA and Nistari breeds, while in other two breeds only traces are found. The amount of hydroxyl amino acids is more in multivoltines than among bivoltines. Of the basic amino acids, lysine is present in all the four breeds, while notable amount of arginine is present in NB_4D_2 and histidine in C. nichi, but in all other breeds only trace amount is recorded. Significant quantity of methionine is present in bivoltines, while only trace amount in multivoltines, and cysteine is present in all the breeds. The proline is also present in all the breeds but in trace quantity.

The crude enzyme was used to isolate and purify by column chromatography. The bivoltine yielded about 750 mgs, whereas multivoltine about 670 mgs of enzyme for every gram of crude sample loaded onto DEAE.-Cellulose column (Table 1). Such a relationship of more protein in bivoltines and less in multivoltines bears direct correlation with the texture of cocoons. The fact that the cocoonase binds to DEAE-Cellulose column indicates that the crude enzyme is rich in acidic amino acids. Further it is supported by the results of amino acid analysis as acidic amino acids are more in number.

Research Paper **© 2012 IJFANS. All Rights Reserved**

The analysis of electrophoretic mobility of bound protein from DEAE-Cellulose column and that of Fraction-II of sephadex gel filtration displayed similarity in molecular weight. The present study indicated that the cocoonases of Bombyx mori is a glycoprotein, possessing a glucose-like carbohydrate. However, further investigations are needed to establish role of carbohydrates in cocoonase activity.

The amino acid analysis of purified cocoonase showed that the acidic amino acids like glutamic acid and aspartic acid are relatively more (Table 5), which is in conformity with the result obtained by ion exchange chromatography. The amount of aromatic amino acids are very less and tryptophane is found to be completely absent in all the four breeds.

With the knowledge gathered in these investigations, it is expected that the cocoonase enzyme can be applied as an anti crease factor since it is involved in the digestion of sticky sericin. Such an approach, in the long run, may pave the way to revolutionize the silk industry, especially during reeling, weaving and dyeing stages.

ACKNOWLEDGEMENTS

Authors wish to thank University of Mysore, Mysore and Indian Institute of Science, Bangalore for extending the facilities to carry out this work.

REFERENCES

[1]. F. Duspiva, The enzymatic processes when the silk spinner (Bombyx mori L.) breaks through the cocoon shell. Journal of Natural Science, Biology and Medicine, 1950, 5: 273 281.

[2]. F.C. Kafatos., A.M. Tartakoff and J.H. Law, Cocoonase I. Preliminary characterization of a proteolytic enzyme from silk moths. Journal of Biological Chemistry, 1967, 242(7): 1477-1487.

[3]. F.C. Kafatos and N. Feder, Cytodifferentiation during insect metamorphosis: the galea of silk moths. Science,1968, 161: 470-472.

[4]. F.C. Kafatos, J.H. Law and A.M. Tartakoff, Cocoonase II. Substrate specificity, inhibitors and classification of the enzyme. Journal of Biological Chemistry. 1967, 242(7): 1488-1494.

[5]. R.L. Felsted, J.H. Law, A.K. Singh and M.S. Jolly, Properties of Antheraea mylitta cocoonase. Comparative Biochemistry and Physiology,1973A, 44B: 595-609.

[6]. P. Andrews, The gel filtration behavior of proteins related to their molecular weights over a wide range. Biochemical Journal,1965, 96(3): 595-606.

[7]. J.F. Hruska and J.H. Law, Methods in Enzymology. Academic Press, New York, 1970.

[8]. R.L. Felsted, K.J. Kramer, J.H. Law, E. Berger and F.C. Kafatos, Cocoonase IV. Mechanism of activation of prococoonase from Antheraea polyphemus. Journal of Biological Chemistry. 1973B, 248(9): 3012-3020.

. [9]. E. Berger, F.C. Kafatos, R.L. Fe1sted and J.R. Law, Cocoonase III. Purification, preliminary characterization and activation of the zymogen of an insect protease. Journal of Biological Chemistry, 1971, 246(13): 4131-4137

[10]. K.J. Kramer, R.L. Felsted and J.H. Law, Cocoonase IV. Structural studies on an insect serene protease. Journal of Biological Chemistry,1973, 248(9): 3021-3028.

[11]. S. Krishnaswami, New technology of silkworm rearing. Central Sericultural Research and Training Institute Bulletin No.2, Central Silk Board, India, pp. 1-23, 1978.
[12]. B.A. Parthasarathi, Isolation, purification and characterization of cocoonase from a few races of Bombyx mori L., Ph.D.Thesis, University of Mysore, Mysore, India, 1993.
[13]. O.H. Lowery, N.J. Rosebrough, A.L. Favor and R.J. Remdall, Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry,1951, 193: 265-275
[14]. B.J. Davis, Disc Electrophoresis II. Methods and application to human serum proteins. Annals of the New York Academy of Science, 1964, 121: 404-427
[15]. K. Weber and M. Osborn, The reliability of molecular weight determination by dodecyl sulphate - poly acrylamide gel electrophoresis. Journal of Biological Chemistry, 1969, 244(16): 4406-4412.
[16]. S. Moore and W.H. Stein, Methods in Enzymology. Academic Press, New York, 1963.--