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Identification of Diapause and Non-diapause Associated Proteins in the Eggs of Multivoltine Silkworm *Bombyx mori* by MALDI MS Analysis

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The diapause and non diapause associated proteins of multivoltine silkworm eggs were analysed by two dimensional (2D) gel electrophoresis. The study was made at 0 hr, 24 hrs and 48 hrs after oviposition. A total of four protein spots in diapause eggs at 24 hrs of oviposition and two protein spots in non diapause eggs at 0 hrs of oviposition were observed. All the six protein spots were considered to have association with diapause and non diapause characters. The molecular weight (MW) and isoelectric point (P*I*) of these 6 protein spots were calculated. The protein spots 1 and 2 observed in 0 hr of non diapause eggs were found to have the MW of 67 and 75 KDa and P*I* of 8.6 and 8.4 respectively. Similarly the four protein spots observed in diapause egg at 24 hrs of oviposition exhibited MW viz., 15, 17, 20 and 25 KDa and P*I* of 5.3, 5.8, 6.5 and 6.0 respectively. All these 6 identified protein spots were subjected to in-gel digestion and resulted tryptic peptides were analyzed by Matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI TOF-MS). Databases searched based on experimentally determined molecular weights of peptides for the determination of the identities of proteins. The identified proteins indicated homology of 34% to 95%. The results indicate that the proteins may play a role in development of diapause and non diapause eggs.

Key words: Diapause proteins, silkworm, *Bombyx mori,* Isoelectric focusing (IEF), MALDI MS.

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Introduction

In insects, diapause strategy is adopted for survival under unfavorable environmental conditions. In most of the species development was arrested and diapause is initiated at a specific stage (Nijhout, 1994). In the case of *Bombyx mori*, diapause occurs at the late gastrula stage of embryogenesis (Nakagaki *et al.*, 1991). The diapause mechanism is initiated by the diapause hormone, which is secreted by the suboesophageal ganglion located in the female pupal head which is transferred at the time of ovarian development (Sato *et al.*, 1993). During the process of diapause initiation and termination it was reported that a number of proteins are synthesized.

Dorel and Coulan (1988) have analysed the expression patterns of proteins synthesized in pre diapausing eggs of *B.mori* by two-dimensional gel electrophoresis using labeled proteins. They identified a protein, which is specific for 24 hrs after oviposition and over produced in the HCL treated eggs. But biological function of the protein is unknown. In contrast to this, two diapause-associated proteins were identified which occurs at only early embryonic stage under the control of diapause down regulated gene (Go *et al.*, 2004). A diapause-associated protein was electrophoretically isolated from the haemolymph of diapausing late age larvae of the pink bollworm *Pectinophora gossypiella.* It was reported that the concentration of diapause protein increased dramatically in the haemolymph of diapausing larvae. This suggests active release of proteins from the fat body into the haemolymph during the development of diapause (Salama and Miller 1992). Jung *et al.*, (2005) identified a diapause associated protein from two spotted spider mite, *Tetranychus urticae.*

They found 25 proteins that have higher quantity in diapause were selected at the range of low molecular weight (5-20 K KDa). Among them 13 proteins were only present in diapause mites and this indicates that some proteins were expressed during diapause and may have specific roles during long over wintering period. Morotomi *et al.*, (2004) analysed the diapause egg of *Triops longicaudatus*, using two-dimensional gel electrophoresis and two protein spots (26 Kda, 4.7 isoelectric point) appeared only in diapause eggs, which have some relationship with long diapause.

The protein 61 (P61; 61 kDa) synthesized only in the diapausing gastrula stage of *B. mori*. The synthesis of P61 was decreased in the eggs kept at 5°C and after the artificial prevention of diapause. Thus, P61 can be described as a diapause arrest-associated protein (Coulon and Dorel, 1991). The *Busseola* diapause protein, (BDP), identified from haemolymph of diapausing larvae of the stem borer, *Busseola fusca* could serve for storage function by providing the amino acids needed for the synthesis of pupal and adult structures (Osir *et al.*, 1989). BDP is induced only during diapause and may play an important role during diapause by binding and transporting juvenile hormone (JH) within the haemolymph (Osir *et al.*, 1991). Spruce budworm larvae produce large quantities of two proteins viz., *Choristoneura fumiferana* diapause associated proteins CfDAP1 and CfDAP2 which are diapause related. These proteins appeared soon after hatching and increased in abundance, reaching maximum levels by four days in the 1st instar, and remained at high levels until three days after the termination of diapause (Palli *et al.*, 1998). The expression of *Cf*DAP and *Cf*DAP-like proteins is associated with starvation stress imposed either experimentally or by elements of the diapause (Han *et al.*, 2003).

Rates of protein synthesis are substantially depressed in diapause II embryos of *Austrofundulus limnaeus*. The major depression of protein synthesis during diapause II affords considerable reduction in energy demand and extends the duration of dormancy attainable in these embryos (Podrabsky and Hand, 2000). The DAP accumulated in the fat body of diapausing larvae of the southwestern corn borer, *Diatraea grandiosella,* at the beginning of diapause, reached a plateau, and gradually declined towards the end of diapause. Similarly, the DAP in last instar non-diapausing larvae treated with JH analogue increased, reached a plateau and then declined (Dillwith *et al.*, 1985).

A low molecular weight diapause-associated protein accumulates in the fat body of last instar pre-diapausing larvae of the southwestern corn borer, *Diatraea grandiose.* Since substantial amounts of the protein accumulate in the fat body before diapause begins, this protein serves as a storage molecule. The specific function of the protein not determined (Dillwith and Chippendale, 1984). The three diapause-associated transcripts (DAT-1, 2 and 3) were isolated from the Colorado potato beetle, *Leptinotarsa decemlineata.* The expression of DAT-1 and 2 are first detected in day 3 and DAT-3 begins on day 12 in diapause programmed adults. Expression of DAT-1, 2 and 3 continues at least 60 days into diapause (Yocum, 2003). Wang *et al.* (2007) reported on early molecular responses of encysted gastrula of brine shrimp Artemia's post diapaused cysts and found that dehydrated cysts actually store more proteins. They traced nine differentially expressed proteins (COXI, COXIII, heat shock proteins (HSP26, HSP60, and HSP70), CDC48, late embryogenesis abundant (LEA), GS1-like protein, and cathepsin L-associated protein (CLAP)) and reported these proteins exhibit distinct expression patterns that suggest complex gene regulations for cyst reactivation after diapause breakage.

Recently, MALDI-MS has been successfully applied for the detection of a number of intact non covalent complexes, such as protein quaternary structures (Rosinke *et al.*, 1995), metal-peptide complexes (Woods *et al.*, 1995), complementary DNA strands (Lecchi and Pannell 1995) and specific complexes of basic peptides with oligo deoxyribonucleotides (Tang *et al.*, 1995). Electrospray ionization mass spectrometry has also been proven a general method for the analysis of a variety of non covalent structures. MS/MS combined with database search methods can identify the proteins present in complex mixtures. A glycoprotein, esterase TIME-EA4 was isolated from diapause eggs of the silkworm, *B. mori* (Pitchayawasin *et al.*, 2004). The protected form of the sugar unit of glycopeptide TIME-EA4, that is isolated from the diapausing eggs of the silkworm, *B. mori* (Hiro *et al.*, 2006). The electrospray ionization (ESI)-tandem quadrupole/orthogonal-acceleration time-of-flight (Q-TOF) mass spectrometer combined with the nano-HPLC system was utilized to determine the glycosylation site and the glycan structure in glycoprotein TIME-EA4 from *Bombyx* diapause eggs (Kurahashi *et al.*, 2002). In the present study, diapause associated protein pattern was analyzed in non diapause and diapause induced eggs of multivoltine silkworm.

Materials and methods

Experimental strain

A multivoltine (non diapause) strain WAI-4 was selected for the study. The larvae were reared by standard rearing method (Krishnaswamy, 1978). The diapause eggs were

obtained by late stage (4^{th} & 5^{th} instars) larvae reared under low temperature (18°C) and photoperiod (6L : 18D) till spinning (Saravanakumar and Ponnuvel 2007) and subsequent eggs were prepared at 25°C.

Sample preparation

The diapause and non diapause eggs were collected after oviposition up to 3 days at 6 hour intervals. 100 mg of eggs were homogenized with 500 µl of phosphate buffered saline (PBS) and the soluble protein was isolated by centrifugation at 10,000 g for 10 min at 4°C. The supernatant protein was removed and stored at −20°C as aliquot. The protein concentration was determined with bovine serum albumin (BSA) as a standard (Bradford, 1976).

SDS PAGE

SDS - polyacrylamide gel electrophoresis was performed using standard methods on the 8 cm mini gels (Laemmli, 1970). 10 µl of protein sample was boiled for 5 min to denaturation and electrophoresed on 10% acrylamide gels of 1 mm thickness. Electrophoresis was carried out with an initial voltage of 35 for 30 min followed by 75 V for 3 hrs until the tracking dye reached the bottom of the gel. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid for 1 h and destained overnight with the destaining solution containing 30% methanol and 10% acetic acid.

Two-dimensional gel electrophoresis

The 2D gel electrophoresis was performed by standard protocol (O' Farrel, 1975). 150 µg of protein sample was added to sample buffer containing 9M urea, 4% NP-40, 2% carrier ampholytes (pH 3 - 10) (Amersham Pharmacia Biotech Ltd),10% DTT, 0.002% bromophenol blue (BPB). Isoelectric focusing (IEF) was carried out in tube gels (PH 3 - 10) system with pre run at 200 V for 30 min followed by separation at 400 V for 3 hrs and end of run at 500 V for 30 min using 10 mM phosphoric acid as anolyte and 10 mM NaOH as catholyte. After IEF the focused tube gels were subjected to additional reduction and alkylation treatment before the second dimension SDS-PAGE. The tube gels were equilibrated in equilibration solution (125 mM Tris HCl , 2% SDS, 10% glycerol, 4.9 mM DTT, BPB traces, pH − 6.8) for 20 min. For the second dimension, the focused tube gels were loaded on 12.5% SDS gels and run with SDS buffer at 30 V for 30 min followed by 75 V for 3 hr until the dye reached the end.

Silver Staining and spot detection

Silver staining was performed as per the method of Blum *et al.* (1987), after electrophoresis the gel slab was fixed in 50% methanol and 10% acetic acid in water for 30 min. It was then incubated for 15 min with 5% methanol in water and additionally for 15 min with water to remove the remaining acid. The gel was sensitized by 2 min incubation in cold 0.02% of sodium thiosulfate and it was then rinsed with three changes of distilled water for 30 sec each. After rinsing, the gel was submerged in cold 0.2% silver nitrate solution and incubated for 25 min at 4°C. After incubation, the silver nitrate was discarded, and the gel slab was rinsed with three changes of water for 1 min each and then developed in 0.04% formalin (35% formaldehyde in water) in 3% sodium carbonate with intensive shaking. After the developer turned yellow, it was discarded and replaced with fresh portion. After the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with 1.5% EDTA. Silver-stained gels were stored in a solution of 5% acetic acid at 4°C until analyzed.

In gel digestion

The protein spots were excised from the silver stained gels according to the method of Shevchenko *et al.* (1996) and the gel pieces were washed with 50% acetonitrile and 50 mM ammonium bicarbonate and incubated at room temperature for 15 min. The gels were washed two more times until the dye has been completely removed. The gel was dehydrated in 100% acetonitrile for 5 min and then removed the acetonitrile with pipette and completely dried at room temperature for 10 - 20 min in a centrifugal evaporator. The gel pieces were rehydrated with digestion solution containing 50 mM ammonium bicarbonate and 20 mg/ml of trypsin (Sigma, sequencing grade) and digested overnight at 37°C. Peptides were extracted by extraction solution containing 60% acetonitrile and 1% TFA to the gel pieces for 10 min and then removed the supernatant into fresh tube. This step was repeated for one more time. The supernatant containing pooled extracted peptides were dried by centrifugal evaporation to near dryness and then added 5 µl of resuspension solution containing 50% acetonitrile and 0.1% TFA to each tube.

Protein identification by mass spectrometry

The digested spots of interest excised from 2D gels were subjected to MALDI-TOF analysis at Indian Institute of Science (IISc), Bangalore, India. A 0.5 µl of sample and 0.5 µl of alpha cyano-4-hydroxy cinnamic acid matrix (10 mg/ml in 50% acetonitrile and 0.1% TFA) spotted on MALDI plate and allowed the spots to dry completely and then loaded into voyager. All MALDI spectra were calibrated using internal tryptic peaks of 842.5 and

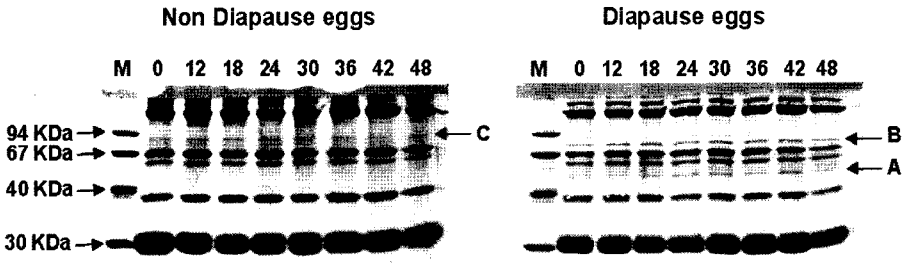

Fig. 1. SDS PAGE patterns of proteins from crude extracts of silkworm eggs using 10% polyacrylamide gels. Non diapause and diapause eggs were incubated at 25°C for 0 to 48 hrs after oviposition. The special protein bands detected in 0 to 48 hrs are shown as A, B and C in non diapause and diapause eggs.

2211.1 Da.

Data analysis

The NCBI nr protein data base was searched first and when no match was found the db EST DNA data base was further searched to determine the identity of the target protein or a protein homologous to the target protein. Mascot (Matrix science) (Perkins *et al.*, 1999) was used to perform data base searching. Mascot a typical peptide mass finger printing program compares the experimentally determined masses of tryptic peptide, with the theoretical masses of all tryptic peptide that can be calculated from sequence of all proteins in the genomic data bases. MS data analyses were performed in a step-wise manner. The *Drosophila* genome data in the NCBI and the silkworm EST data (http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) were employed in the second step of the analysis using Mascot. The proteins matching the input data were listed.

Results

SDS-gel electrophoresis

The electrophoretic pattern of protein of diapause and non diapause eggs were analysed at 6 hr intervals up to 3 days. It was found that non diapause eggs showed special protein band in the range of 83 KDa whereas in diapause eggs this protein was not observed. In diapause eggs a special protein bands in the range of 75 KDa and 48 KDa were found whereas in non diapause eggs, these proteins did not appear (Fig. 1).

2D gel electrophoresis

The 2D gel electrophoretic analysis was performed at 0, 24 and 48 hr of diapause and non diapause egg protein samples based on the results of SDS-PAGE. Non diapause egg of 0 hr showed two special protein spots in the range of 67 and 75 KDa and PI 8.6 and 8.4. These two proteins have very low amount in the subsequent hrs of 24 and 48 hrs and also these proteins were absent in the diapause eggs. In diapause eggs, 24 hr samples showed four special protein spots in the range of approximately 15, 17, 20 and 25 KDa and PI 5.3, 5.8, 6.5 and 6.0 respectively. These proteins were very less amount in the 0 and 48 hr of diapause and also absent in the non-diapause eggs (Fig. 2).

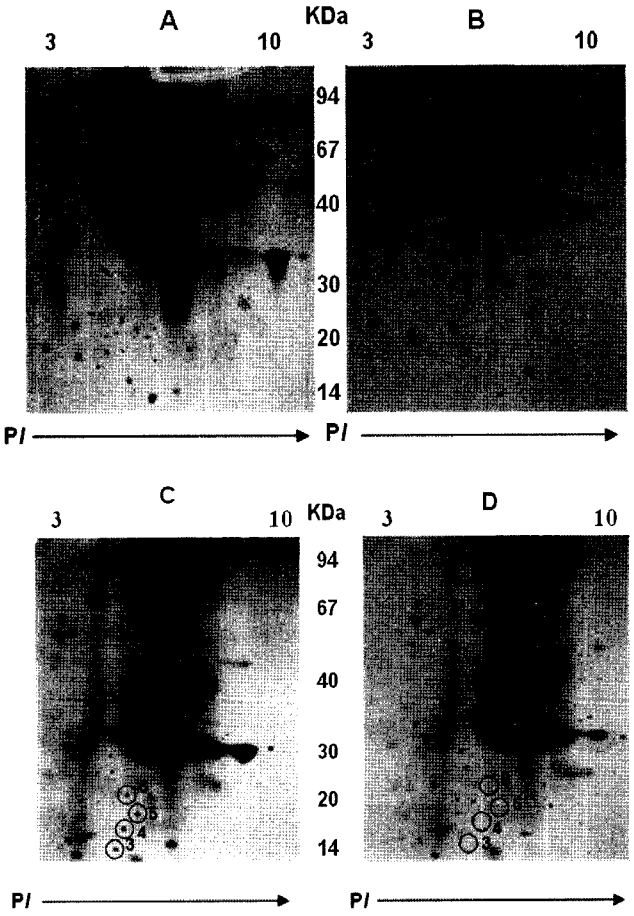

Fig. 2. Silver stained 2D PAGE of protein extracts of A Non diapause and B Diapause eggs after oviposition at 0 hrs. The Diapause C and Non diapause eggs D of 24 hrs after oviposition. All proteins are shown with isoelectric points in the range of pH 3 to 10 and molecular weight in the range of 94 to 14 KDa. The special protein spots are shown as circle and numbered as spot 1, 2 of non diapause and 3, 4, 5 6 of diapause eggs.

Mass spectrometry analysis

The digested peptides of diapause and non diapause were analysed by MALDI MS. The two proteins of non diapause eggs showed the spectra ranged 553.870 to

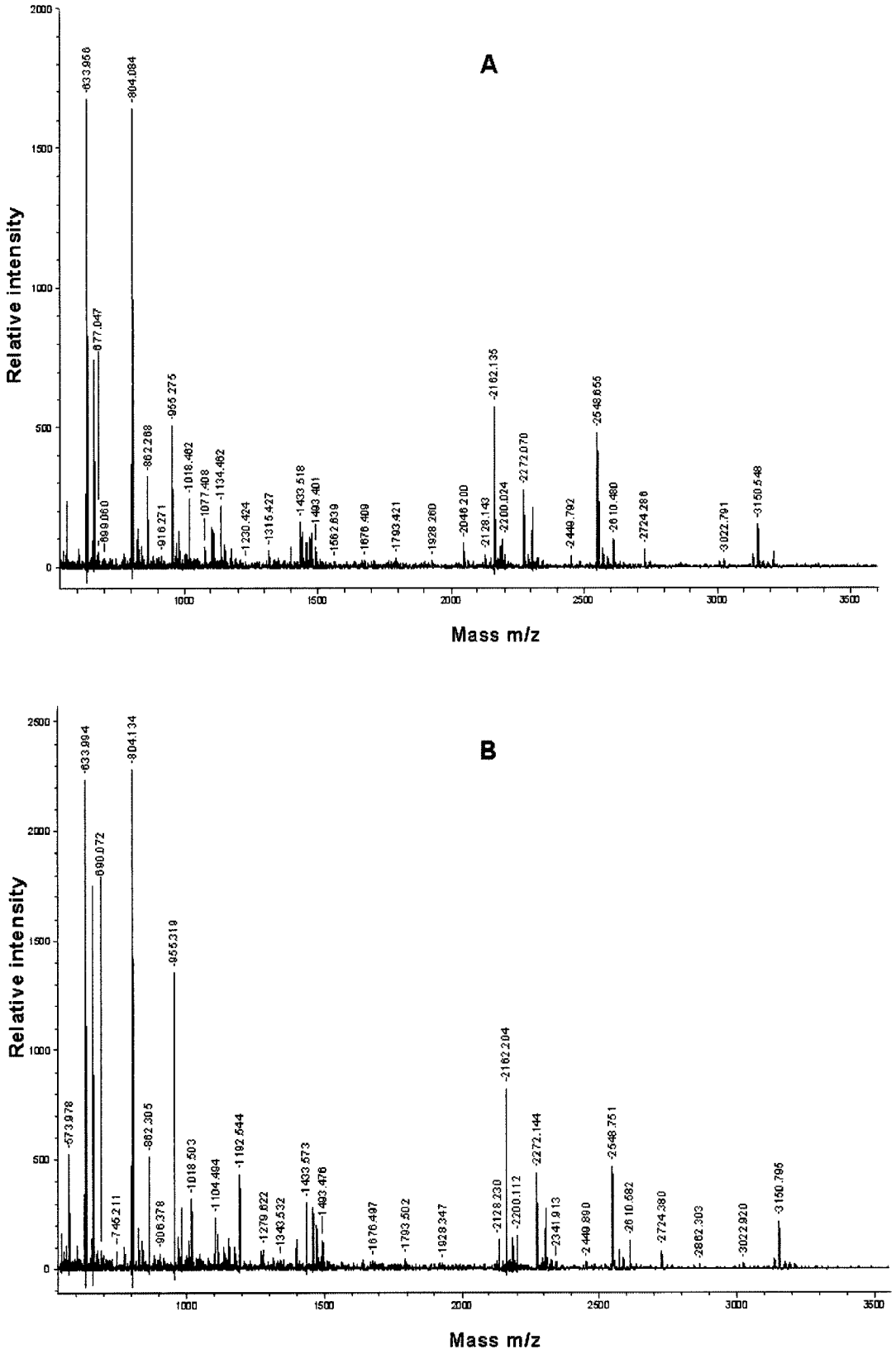

Fig. 3. The MALDI mass spectrum of tryptic peptides detected after the in-gel digestion of the spot 1 (A) and spot 2 (B) of non diapause eggs. In diapause eggs the protein spots 3, 4, 5, 6 are named as C, D, E, and F respectively.

3208.440 m/z with the highest intensity of 1767.92 and spot 2 showed the maximum intensity of 2334.32 and mass ranged from 544.648 to 3172.656 m/z. The protein spot 3 of diapause eggs showed highest intensity 3960.05

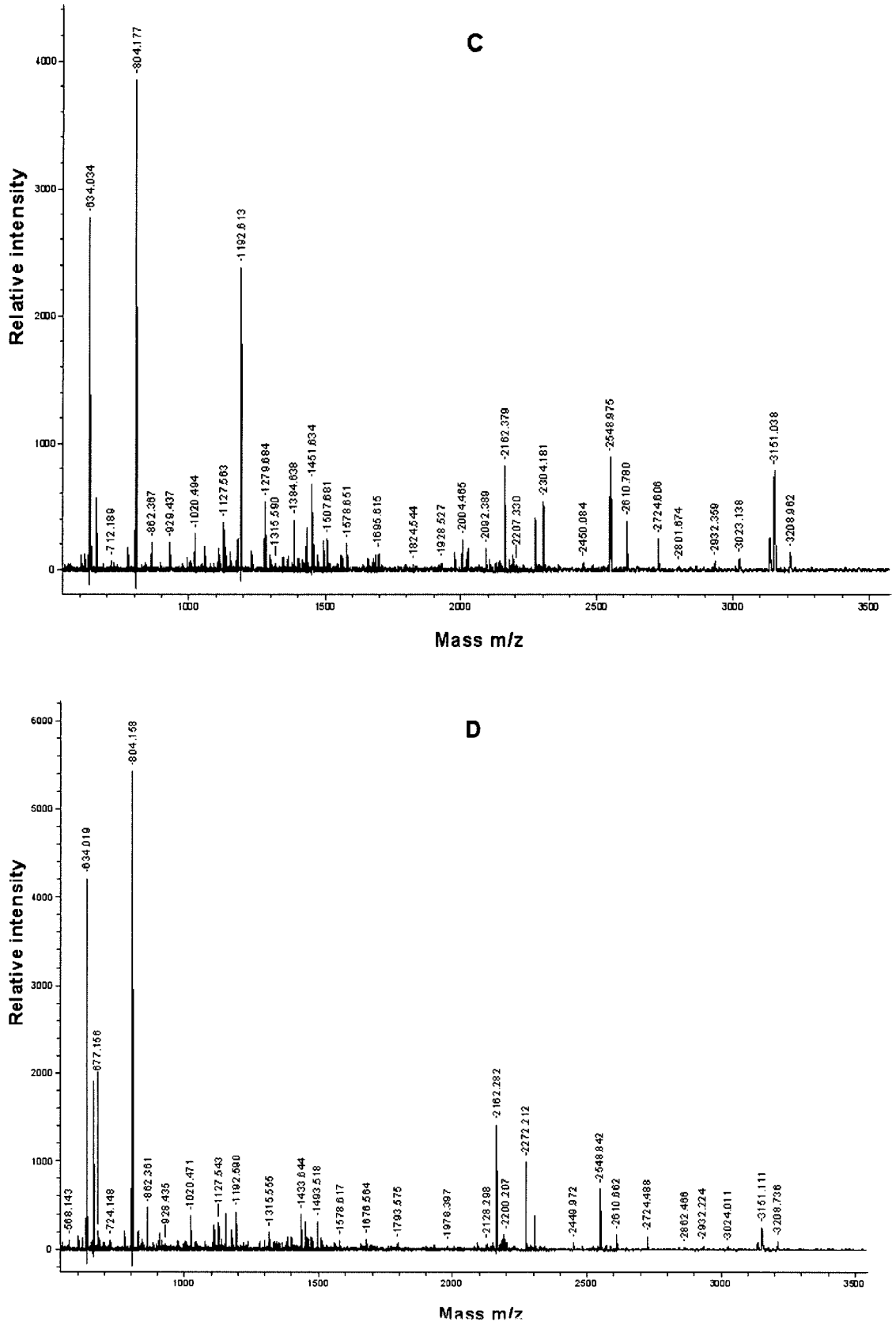

Fig. 3. Continued.

and with the mass of 605.978 to 3208.962 m/z. Similarly protein spot 4 obtained the mass of 544.660 to 3208.736 m/z with highest intensity of 55.15.20. The spot 5 showed mass ranged from 544.677 to 3208.909 m/z with maxi-

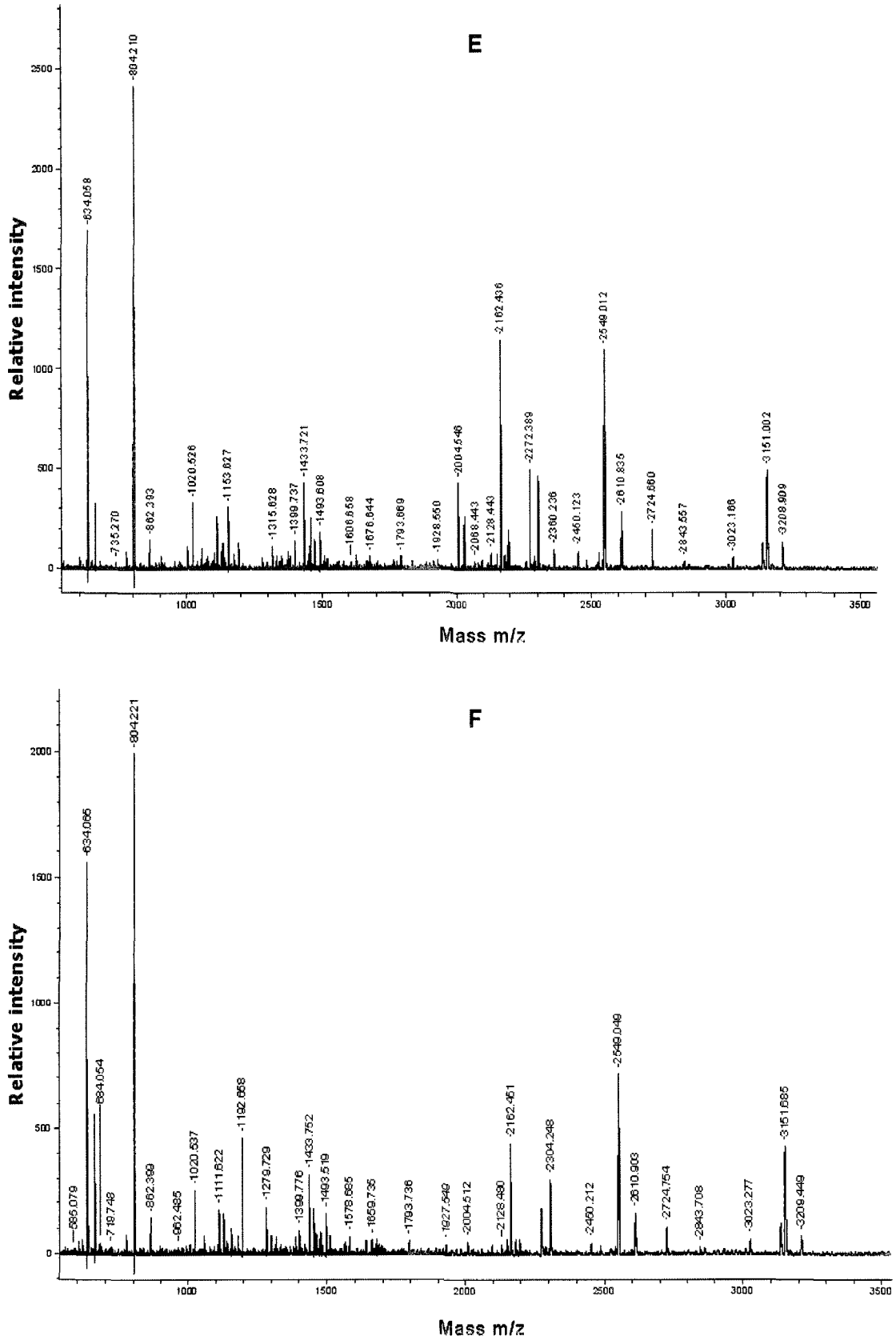

Fig. 3. Continued.

mum intensity of 2505.04 and in spot 6 showed the mass values 553.959 to 3209.449 with the intensity of 2033.53 in diapause eggs (Fig. 3).

Peptide mass fingerprint

The MALDI masses were searched with database for homology. The sequences ranged from 800 to 3000 were entered in the sequence coverage of matching peptide in tryptic peptide mass in the range of 34 to 95% (Table1). The spot 1 matched with protein of Argonaute protein of *Drosophila* with sequence coverage of 34%. The second spot of non diapause eggs showed highest similarities with Helicase RM62-like protein of *Schistocerca gregaria* (Desert locust) and 91% of sequence coverage. The spot 3 showed the homology with the hypothetical protein of *Aspergillus nidulans* FGSC A4 and sequence coverage was matched with 52%. The spot 4 showed similarities with hypothetical protein of *Plasmodium yoelii* at 95% sequence coverage. The diapause eggs of spot 5 data base searched results were matched with Embryonic 1 beta-globin *Danio rerio* (Zebra fish) and sequence coverage was 75%. Similarly the 30K protein (gene 21G1) of silk-worm *B. mori* was matched with 73% sequence of spot 6 in diapause eggs.

Table 1. The data obtained for two non diapause and four diapause associated proteins from two-dimensional PAGE gel spots

Spot No.	MALDI mass	(Measured mass)-(calculated mass)	Peptide sequence determined by PSD and consistent with mass	Protein identified (NCBI Accs No.); Sequence coverage (%) and calculated PI value
1	2303.92	-0.2919	K.RGTIGRPGQVAINYLDLDMSK.M	Acc.No.ABB54744 Argonaute-2 (*Drosophila simulans)* PI value: 8.34 Sequence Coverage: 34%
	971.28	-0.2492	K.IMPERPTK.F	
	2288.04	-1.0450	R.MDQLGGAILAFDGKASCYSVDK.L	
	1136.42	-0.2464	R.TLRYTIEIK.E	
	1104.45	-1.0901	K.ETADSNIDLK.S	
	906.32	-0.1676	R.IFDKPMR.A	
	840.21	-0.2647	R.VGRSFFK.M	
	1480.45	-0.2912	K.INNTTNLEYSRR.F	
	1077.41	-0.2124	R.RFLEPFLR.G	
	922.25	0.7327	R.FLEPFLR.G	
	1399.55	-1.0951	R.APANSEVFEHDGK.K	
	862.27	-0.1771	R.QVSEMIR.F	
	1708.38	0.4229	R.FGIRIANDFIVVSTR.T	
	1142.41	-0.1189	K.WTVLYCDSR.S	
	1793.42	0.5485	R.SGGHKISYNQINDFGR.K	
	1455.48	0.7374	K.ISYNQINDFGRK.I	
	804.08	0.5855	R.KILSQSK.A	
	2200.02	-0.1303	K.AELQHGILTQCVKQFTVER.K	
	2610.48	-0.7445	R.EIPSVVGVAASHDPYGASYNMQYR.L	
	2149.13	0.0789	R.GTLEEIEDMYTVTLEHLR.V	
	1050.35	0.8652	R.DGVSDGQFPK.I	
	1337.42	-0.2324	R.FFPSGVETPSNR.F	
	1785.40	-0.5616	R.SVSYPAPAYLAHLVAAR.G	
	1562.64	-0.2299	R.VYLTGTHRFLDLK.K	
	897.2600	-1.1891	K.KNPMYFV.-	
2	2548.75	0.37	-.QTGSGKTLAYIFPAIVHINHQPR.L	Acc. No. AAO15911 Helicase RM62-like protein B (Fragment). *Schistocerca gregaria* (Desert locust). PI value: 8.04 Sequence coverage: 91%.
	1676.50	-0.48	R.LQRGDGPIALVLAPTR.E	
	1278.62	-1.12	R.GDGPIALVLAPTR.E	
	1279.62	-0.12	R.GDGPIALVLAPTR.E	
	2162.20	1.08	R.ELAQQIQQVASEFGASSLVR.N	
	3150.80	1.19	R.ELAQQIQQVASEFGASSLVRNTCIFGGAPK.G	
	1040.47	-1.07	K.GPQARDLER.G	
	1111.49	-0.16	R.GVEIVIATPGR.L	
	906.38	0.87	R.LIDFLER.G	
	818.24	0.77	R.GTTNLRR.C	

Table 1. Continued.

Spot No.	MALDI mass	(Measured mass)-(calculated mass)	Peptide sequence determined by PSD and consistent with mass	Protein identified (NCBI Accs No.); Sequence coverage (%) and calculated PI value
3	1399.70	-0.05	M.FAARQSFGFLQK.R	Acc. No. XP_664321 Hypothetical protein AN6717.2 (*Aspergillus nidulans* FGSC A4) *PI* value: 6.93 Sequence coverage: 52%
	1111.57	0.96	R.QSFGFLQKR.A	
	1793.63	0.54	K.VAVLGASGGIGQPLSLLLK.L	
	2272.31	-1.06	K.VAVLGASGGIGQPLSLLLKLNPR.V	
	1659.64	0.72	K.LNPRVSELALYDIR.G	
	1451.63	0.93	K.GYEPTESGLADALK.G	
	1978.45	0.32	K.GSEIVLIPAGVPRKPGMTR.D	
	992.53	-0.86	K.EDDCENLR.I	
	2724.61	0.12	K.ASPEANILVISNPVNSTVPIVSEVFK.A	
	1091.49	-0.08	R.IQFGGDEVVK.A	
	1665.65	-0.16	K.AKDGAGSATLSMAMAGAR.F	
	2283.31	0.19	K.DGAGSATLSMAMAGARFAESLLR.A	
	1418.62	-1.14	R.FAESLLRAAQGEK.G	
	2149.40	0.27	R.AAQGEKGVVEPTFVESPLYK.D	
	1564.65	-0.17	K.GVVEPTFVESPLYK.D	
	2106.38	-0.66	K.DQGVNFFASKVELGPNGAEK.I	
4	1279.68	1.01	M.EKSNYNSPLVK.G	Acc. No XP_724084 Hypothetical protein PY03868 *Plasmodium yoelii* 17XNL] *PI* value: 5.04 Sequence coverage: 95%
	1020.47	-1.06	K.SNYNSPLVK.G	
	1022.43	0.90	K.SNYNSPLVK.G	
	2162.28	-0.77	K.GDYLSNSYALDFESEIVLK.I	
	2548.84	-0.38	.IPEDATFVRCYVSYVSSIEADGK.R	
	1676.56	-0.23	R.CYVSYVSSIEADGKR.N	
	1451.60	-0.18	K.LEGIGYTDPFIVK.E	
	2586.76	0.49	K.LEGIGYTDPFIVKECHVYPYSR.C	
	1153.58	0.07	K.ECHVYPYSR.C	
	1384.59	-0.02	K.ECHVYPYSRCK.L	
	1045.48	0.95	K.LNTAAGAQELG.-	
5	1153 .63	-0.93	-.MVVWTDFEK.A	Acc. No NP_001091054 Embryonic 1 beta-globin family member [*Danio rerio*]. *PI* value: 6.81 Sequence coverage: 75%
	1279 .73	1.06	R.CLIVYPWTQR.Y	
	2128 .44	-0 .65	K.FGNLYNAAAILGNPMVAAHGK.T	
	2570 .92	0.53	K.FGNLYNAAAILGNPMVAAHGKTVLK.G	
	1444 .69	-0.09	K.GLELAVKNMDNIK.A	
	2149 .42	0.36	K.NMDNIKATYADLSVLHSEK.L	
	1433 .72	-0.01	K.ATYADLSVLHSEK.L	
	2527 .01	-0.25	K.ATYADLSVLHSEKLHVDPDNFR.V	
	1111 .60	-0.95	K.LHVDPDNFR.V	
	1298 .67	0.02	K.LHVDPDNFRVS	
6	3151.68	1.11	-.MKFLVVFASCVLAVSAGVAEMSAVSMSSSNK.E	Acc. No CAA38531. 30 K protein (*Bombyx mori*) *PI* value: 6.33 Sequence coverage: 73%
	1686.65	-0.18	K.LYNSILTGDYDSAVR.Q	
	2862.59	-0.78	K.LYNSILTGDYDSAVRQSLEYENQGK.G	
	2843.71	-0.69	K.GSIIQNVVNNLIIDGSRNTMEYCYK.L	
	1278.73	0.02	K.LWVGNGQHIVR.K	
	1279.73	1.02	K.LWVGNGQHIVR.K	
	992.54	-0.02	R.LIMAGNFVK.L	
	836.26	0.79	R.NYNLALK.L	
	962.48	-0.01	K.NSDLISWK.F	
	1192.66	0.05	K.FITLWENNR.V	
	1421.65	-0.10	K.IHNTKYNQYLK.L	
	2149.47	-0.51	K.YNQYLKLSSTTDCNTQDR.V	
	1295.64	-0.02	R.VIFGTNTADTTR.E	
	1176.63	0.02	R.EQWFLQPTK.Y	
	2541.03	0.83	R.MAFGHDGEVAGLPDIFSWFVTPF.-	

Discussion

Many researchers have reported proteomic analysis by combined two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Mass spectrometry, with hemolymph of *D. melanogaster* (Vierstraete *et al.*, 2003), hemolymph of *Anopheles gambiae* (Paskewitz and Shi, 2005) and of *Boophilus microplus* (Untalan *et al.*, 2005). In the present study, a total of six proteins were identified in diapause and non diapause eggs. Out of 6 proteins, two proteins may be responsible for the non diapause. Dorel and Coulon (1988) analysed proteins synthesis in pre diapausing eggs of *Bombyx* from the fertilization to the onset of diapause. A set of proteins with molecular weight range of 68,000 to 74,000 and isoelectric points of 5.85 - 5.95 is specific of the germ-anlage stage. Transcripts encoding the proteins are not detectable in oocytes. When treating eggs at the germ-anlage stage with 4 N HCl at 46°C prevents diapause is accompanied by overproduction of protein. This implication is related to early embryonic development and prevention of diapause.

In database search, a result of protein spot 1 was found to have homology with the *Drosophila* argonaute protein. Argonaute proteins were found in all higher eukaryotes and have important functions in processes as diverse as embryonic development, cell differentiation and transposon silencing (Höck and Meister 2008). Similarly in protein spot 2 of non diapause eggs, 91% sequence matched with helicase like protein (RHL). RHL has an important role in the early embryo of the silkworm *B. mori* and the expression of the RHL mRNA is associated with non diapause or diapause termination development process (Sawada *et al.*, 2006).

Spot 3 and 4 showed high similarities with hypothetical protein of *Aspergillus nidulans* and *Plasmodium yoelii.* The specific functions of these proteins are unknown. A two proteins spot with an approximate molecular weight of 38 KDa and P*I* 6.1 was identified in diapause eggs and suggested that this protein may be associated with the onset of diapause character. Go *et al.*, (2004) observed a spot with an approximate molecular weight of 21 KDa and P*I* of 6.1, which is considered to be a diapause specific protein and occurred only at early embryonic stage.

Many proteins isolated and identified from diapause and non diapause eggs of the silkworm, *B. mori.* TIME-EA4 is an ATPase that measures time intervals, functioning as a diapause duration clock in diapause eggs. The sequence of TIME-EA4 has been established as that of a metallo-glycoprotein by combinational means involving peptide sequence analysis, nano-HPLC-ESI-Q-TOF-MS and MS/MS. The amino acid sequence of TIME-EA4 showed 46-55% homology with the reported proteins of the Cu, Zn-SOD (superoxide dismutase) family and the structures are identical in *Bombyx* SOD, bovine SOD, and TIME-EA4 proteins (Isobe *et al.*, 2006).

Spot 6 of diapause eggs showed 73% sequence homology with *Bombyx* 30 K protein. Similar results also reported by Sawada *et al.* (2007) and they studied the relationship between Ommin-binding protein (OMBP) and the 30 K proteins, which were localized in yolk granules and serosa in the diapause egg of *B. mori.* In addition, they confirmed the expression of 30 K proteins mRNA at early embryonic stage in diapause eggs by RT-PCR analysis. The 30 K proteins as OMBP may play an important role in the transport and accumulation of tryptophan metabolites and ommochrome during the formation of serosa.

In conclusion, there were at least six unique protein spots identified in the silkworm of WAI-4 strain. These proteins with similar molecular sizes and isoelectric points were matched with the earlier reported protein, which is responsible for the diapause character. Therefore these protein variations in the diapause and non diapause eggs may have a role in embryo developmental or diapause initiation and termination process. The protein analysis remains essential in the field of post-genome analysis and is expected to contribute for a better understanding of specific functions of proteins involved in the diapause and non diapause eggs.

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