CHAPTER IV

RESULTS AND DISCUSSION

Amplification and cloning of *Tribolium castaneum* α-amylase gene (TcAm)

The α-amylase genes have been studied in several insects. Numerous reports showed that insect α-amylase genes are approximately 1,400-1,700 bp in length such as *Zabrotes subfasciatus*: 1,449 bp (Grossi and Chrispeels.,1997), *Scirpophaga incertulas*: 1,506 bp (Sharma, 2009), and *Sitophilus oryzae* : 1,485 bp (Lertkaeo, 2011). According to our study, the 430 bp of insect β-actin gene and 1,650 bp of *Tribolium castaneum* α-amylase gene were amplified (Figure 16) using specific primers designed based on *Tribolium castaneum* α-amylase mRNA sequence, which consists of 1,581 nucleotides deducing to 490 amino acid residues (NCBI Reference Sequence: NM_001114376.1). All PCR reactions showed a single DNA band, which differs from the*Tribolium castaneum* α-amylase mRNA sequence in the database. The size of bands observed in this study was greater than reported *Tribolium castaneum* α-amylase mRNA sequence. The total RNA average ratio (OD 260/280) was in the range of 1.8–2.0, indicating that the RNA was at high quality.

The 1,650 bp of α-amylase cDNAs were isolated from agarose gel, and cloned by TA-cloning system. The ligated products were transformed into *E.coli* (DH5α). Thirteen cDNA clones of 1,650 bp were obtained. Recombinant clones were screened with colony PCR and *BamHI* and *XhoI* restriction enzyme analysis (Figure 17 and Figure 18). After the isolation of plasmid, the nucleotide sequences were determined.

The 1,650 bp ofTcAm gene sequence analysis

After extensive screening of clones by colony PCR and restriction digestion analysis, thirteen recombinant clones were successfully obtained and sequenced. However, the 1,650 bp of TcAm detected in these thirteen recombinant clones contained four extra regions of, 50 bp, 38 bp, 46 bp and 47 bp (Figure 19B), along with the abnormal stop codon compared to the *T.castaneum* α-amylase mRNA NCBI nucleotide database (NCBI Reference Sequence: NM_001114376.1) (Figure 20 and

Figure 21), which the full-length sequence of *T.castaneum* α-amylase was achieved after their removal. Moreover, the intron splice site of *Tribolium castaneum* genomic sequence (NCBI Gene ID: 663954) was matched at the similar position with the obtained 1,650 bp of TcAm. Therefore, these extra regions could be assumed as the pseudo-genes, since the pseudo-genes of insect amylases have been previously reported such as the α-amylase of *Drosophila pseudoohscura* (Popadic, 1996).

Expression of 1,650 pb recombinant *Tribolium castaneum* α-amylase

The pET32a(+) – 1,650 bp of TcAmrecombinant protein were produced in *E. coli*BL21(DE3)pLysS at 37 °C, 1 and 2 hr by the induction with1 mM IPTG. SDS-PAGE analysis of the pET32a(+) – 1,650 bp and the pET32a(+) control total protein from whole-cell extracts showed the bands at the size of approximately 25 kDa and 18 kDa (Figure 22), respectively. The crude extracts of the pET32a(+) – 1,650 bp and the pET32a(+) control activities were determined by Chloro-4-nitrophenyl-α-D-maltotrioside substrate. The amylolytic activity was not found in both the pET32a(+) – 1,650 bp and the pET32a(+) control. The results indicated that the cells were capable of expressing only the fusion protein in the expression vector and the short protein of TcAm gene (18 kDa Thioredoxin/Hitidine tag from vector and 7 kDa from TcAm gene), according to the cDNA sequence of 1,650 bp TcAm genes that an abnormal stop codon was found within (Figure 21).

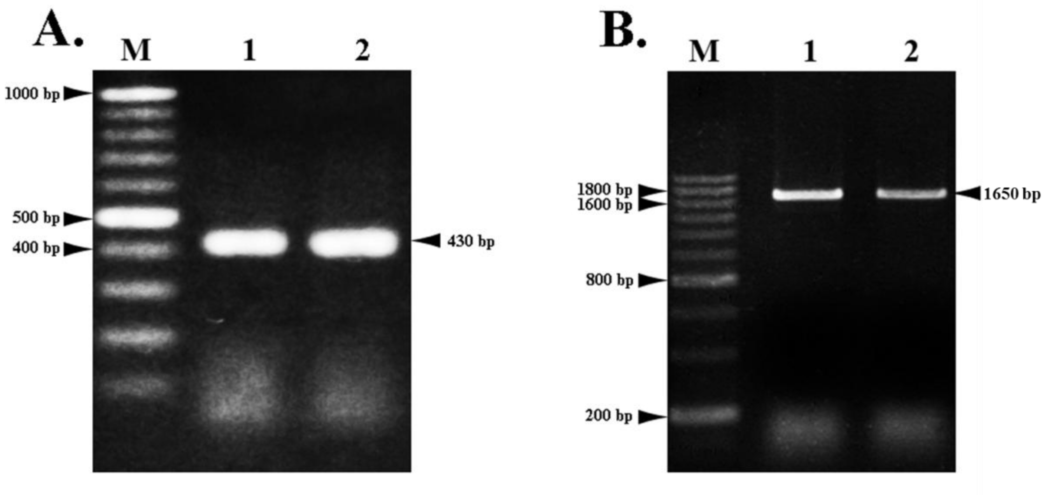


Figure 16 Agarose gel electrophoresis of PCR product
(A) M: 100 bp DNA molecular weight ladder, Lane 1-2: insect β-actin gene.
(B) M: 200 bp DNA molecular weight ladder, Lane 1-2: 1,650 bp *Tribolium castaneum* α-amylase gene

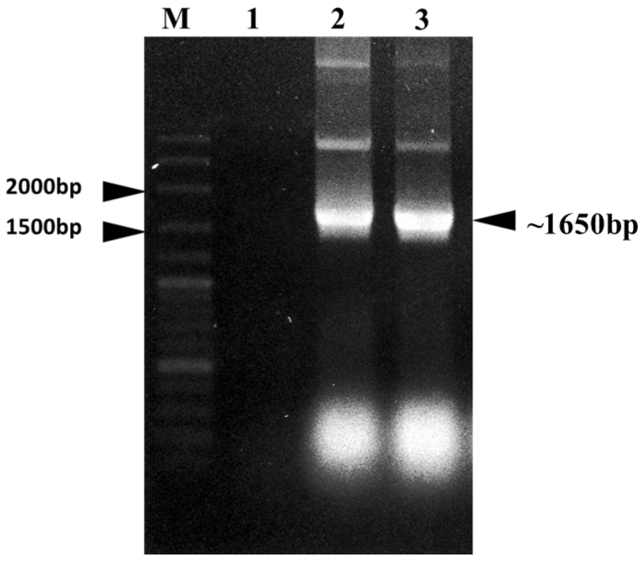


Figure 17 Agarose gel electrophoresis of colony PCR screening of the 1,650 bp TcAm recombinant plasmid with specific primers. M: 100 bp DNA molecular weight Ladder, Lane 1: Negative control, Lane 2-3 PCR products of 1,650 bp TcAm

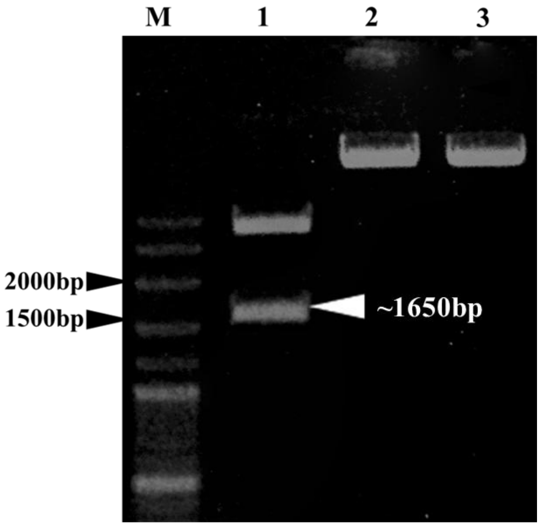


Figure 18 **Agarose gel electrophoresis of *BamHI* and *XhoI* restriction enzyme analysis of 1,650 bp TcAm. M: 100 bp DNA molecular weight Ladder, Lane 1: Recombinant plasmid digested with *BamHI* and *XhoI*,Lane 2, Recombinant plasmid digested with *BamHI*, Lane 3: Recombinant plasmid digested with *XhoI***

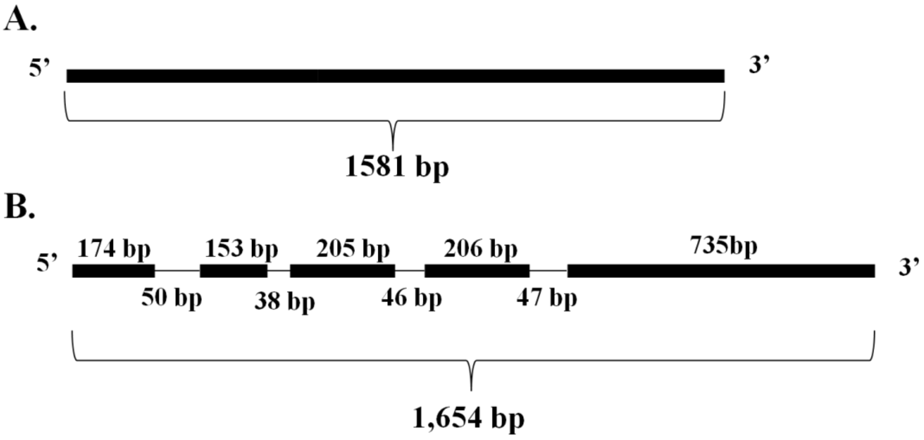
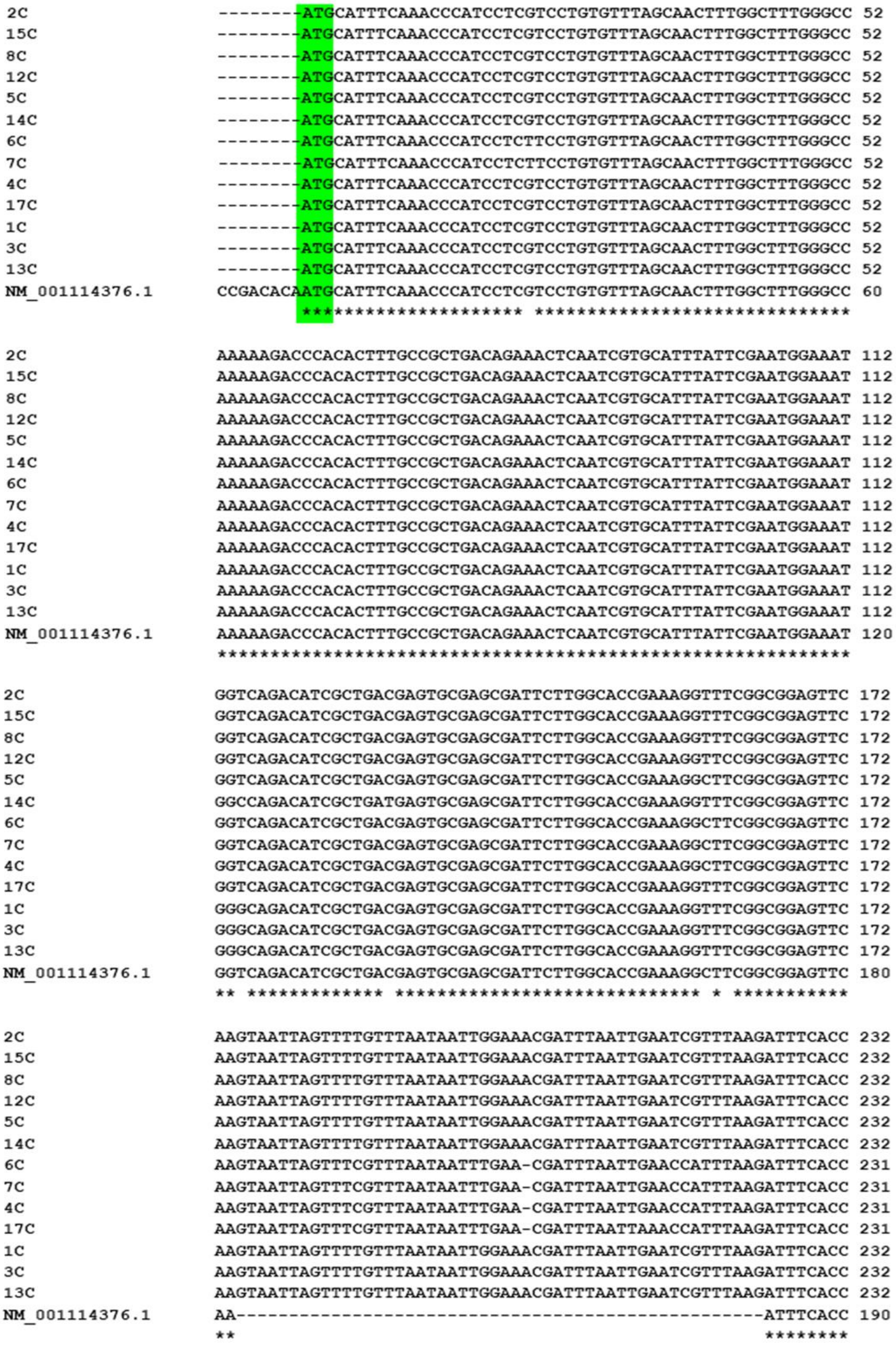


Figure 19 **Diagrams depicting comparison between (A) *Tribolium castaneum* α-amylase mRNA (NCBI Reference Sequence: NM_001114376.1) and (B) TcAm cDNA cloned in this study. The thin lines in (B) represent the 4 extra regions of, 50 bp, 38bp 46 bp and 47 bp with abnormal stop codon in first region.**



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12C                   --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
5C                    --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
14C                   --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
6C                    --------ATGCATTTCAAACCCATCCTCTTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
7C                    --------ATGCATTTCAAACCCATCCTCTTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
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17C                   --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
1C                    --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
3C                    --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
13C                   --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTGGCTTTGGGCC 52
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12C                   GGTCAGACATCGCTGACGAGTGCGAGCGATTCTTGGCACCGAAAGGTTCCGGCGGAGTTC 172
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13C                   GGGCAGACATCGCTGACGAGTGCGAGCGATTCTTGGCACCGAAAGGTTTCGGCGGAGTTC 172
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NM_001114376.1        AA--------------------------------------------------ATTTCACC 190
                      **                                                  ********
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Figure 20 **The comparison of 1,650 bpTcAm cDNA cloned nucleotide sequences with *Tribolium castaneum* α-amylase mRNA (NCBI Reference Sequence: NM_001114376.1)**

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12C             GGTGAGTTACATCCTCAATACGCGCTCAGGGGACGAAGCGGCTTTGGCTGACATGATCAG 352
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12C             CAGGATTTACGTAGATACTGTCATCAACCACATGACTGGGATGGGCGGTACTGGGACCGC 471
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Figure 20 (Cont.)

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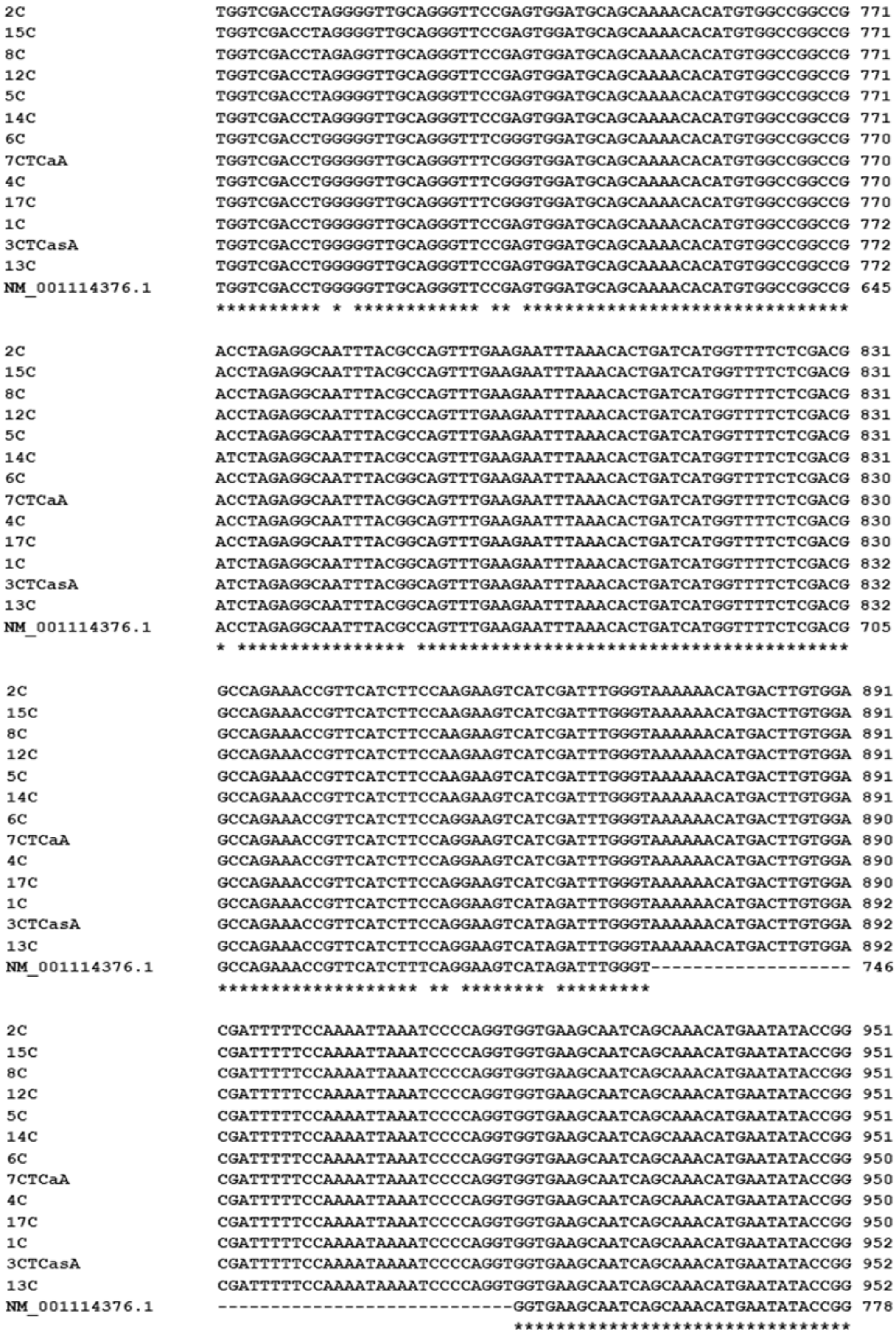
Figure 20 (Cont.)



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7CTCaA             TGGTCGACCTGGGGGTTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCG 770
4C                 TGGTCGACCTGGGGGTTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCG 770
17C                TGGTCGACCTGGGGGTTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCG 770
1C                 TGGTCGACCTGGGGGTTGCAGGGTTCCGAGTGGATGCAGCAAAACACATGTGGCCGGCCG 772
3CTCasA            TGGTCGACCTGGGGGTTGCAGGGTTCCGAGTGGATGCAGCAAAACACATGTGGCCGGCCG 772
13C                TGGTCGACCTGGGGGTTGCAGGGTTCCGAGTGGATGCAGCAAAACACATGTGGCCGGCCG 772
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8C                 ACCTAGAGGCAATTTACGCCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 831
12C                ACCTAGAGGCAATTTACGCCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 831
5C                 ACCTAGAGGCAATTTACGCCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 831
14C                ATCTAGAGGCAATTTACGCCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 831
6C                 ACCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 830
7CTCaA             ACCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 830
4C                 ACCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 830
17C                ACCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 830
1C                 ATCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 832
3CTCasA            ATCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 832
13C                ATCTAGAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCTCGACG 832
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5C                 GCCAGAAACCGTTCATCTTCCAAGAAGTCATCGATTTGGGTAAAAAACATGACTTGTGGA 891
14C                GCCAGAAACCGTTCATCTTCCAAGAAGTCATCGATTTGGGTAAAAAACATGACTTGTGGA 891
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1C                 GCCAGAAACCGTTCATCTTCCAGGAAGTCATAGATTTGGGTAAAAAACATGACTTGTGGA 892
3CTCasA            GCCAGAAACCGTTCATCTTCCAGGAAGTCATAGATTTGGGTAAAAAACATGACTTGTGGA 892
13C                GCCAGAAACCGTTCATCTTCCAGGAAGTCATAGATTTGGGTAAAAAACATGACTTGTGGA 892
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8C                 CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 951
12C                CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 951
5C                 CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 951
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7CTCaA             CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 950
4C                 CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 950
17C                CGATTTTTCCAAAATTAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 950
1C                 CGATTTTTCCAAAATAAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 952
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13C                CGATTTTTCCAAAATAAAATCCCCAGGTGGTGAAGCAATCAGCAAACATGAATATACCGG 952
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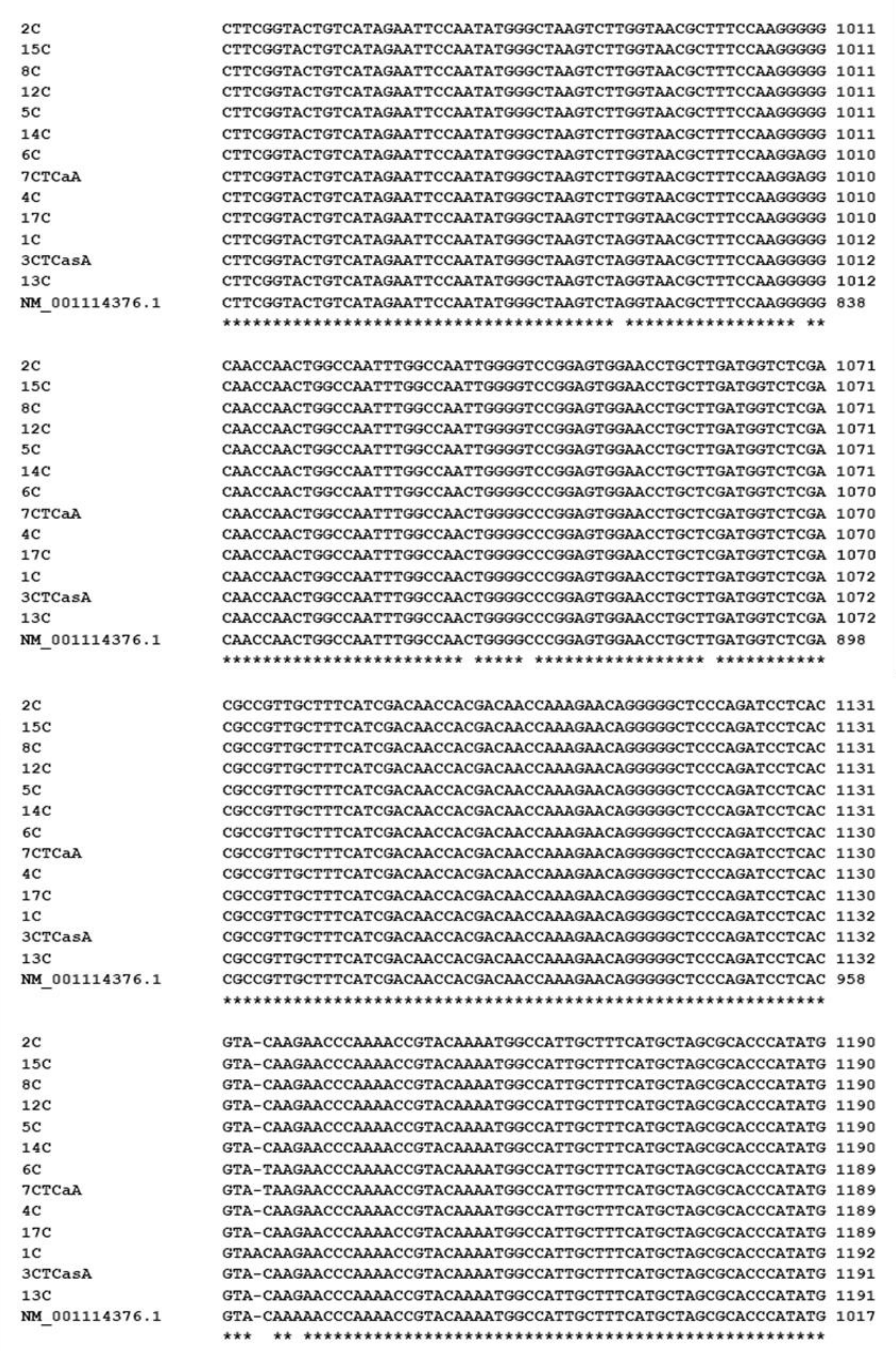
Figure 20 (Cont.)



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12C                 CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGG 1011
5C                  CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGG 1011
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6C                  CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGAGG 1010
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17C                 CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGG 1010
1C                  CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTAGGTAACGCTTTCCAAGGGGG 1012
3CTCasA             CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTAGGTAACGCTTTCCAAGGGGG 1012
13C                 CTTCGGTACTGTCATAGAATTCCAATATGGGCTAAGTCTAGGTAACGCTTTCCAAGGGGG 1012
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12C                 CAACCAACTGGCCAATTTGGCCAATTGGGGTCCGGAGTGGAACCTGCTTGATGGTCTCGA 1071
5C                  CAACCAACTGGCCAATTTGGCCAATTGGGGTCCGGAGTGGAACCTGCTTGATGGTCTCGA 1071
14C                 CAACCAACTGGCCAATTTGGCCAATTGGGGTCCGGAGTGGAACCTGCTTGATGGTCTCGA 1071
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1C                  CAACCAACTGGCCAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTTGATGGTCTCGA 1072
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NM_001114376.1      CAACCAACTGGCCAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTTGATGGTCTCGA 898
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12C                 CGCCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAGATCCTCAC 1131
5C                  CGCCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAGATCCTCAC 1131
14C                 CGCCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAGATCCTCAC 1131
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4C                  CGCCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAGATCCTCAC 1130
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4C                  GTA-CAAGAACCCAAAACCGTACAAAATGGCCATTGCTTTCATGCTAGCGCACCCATATG 1189
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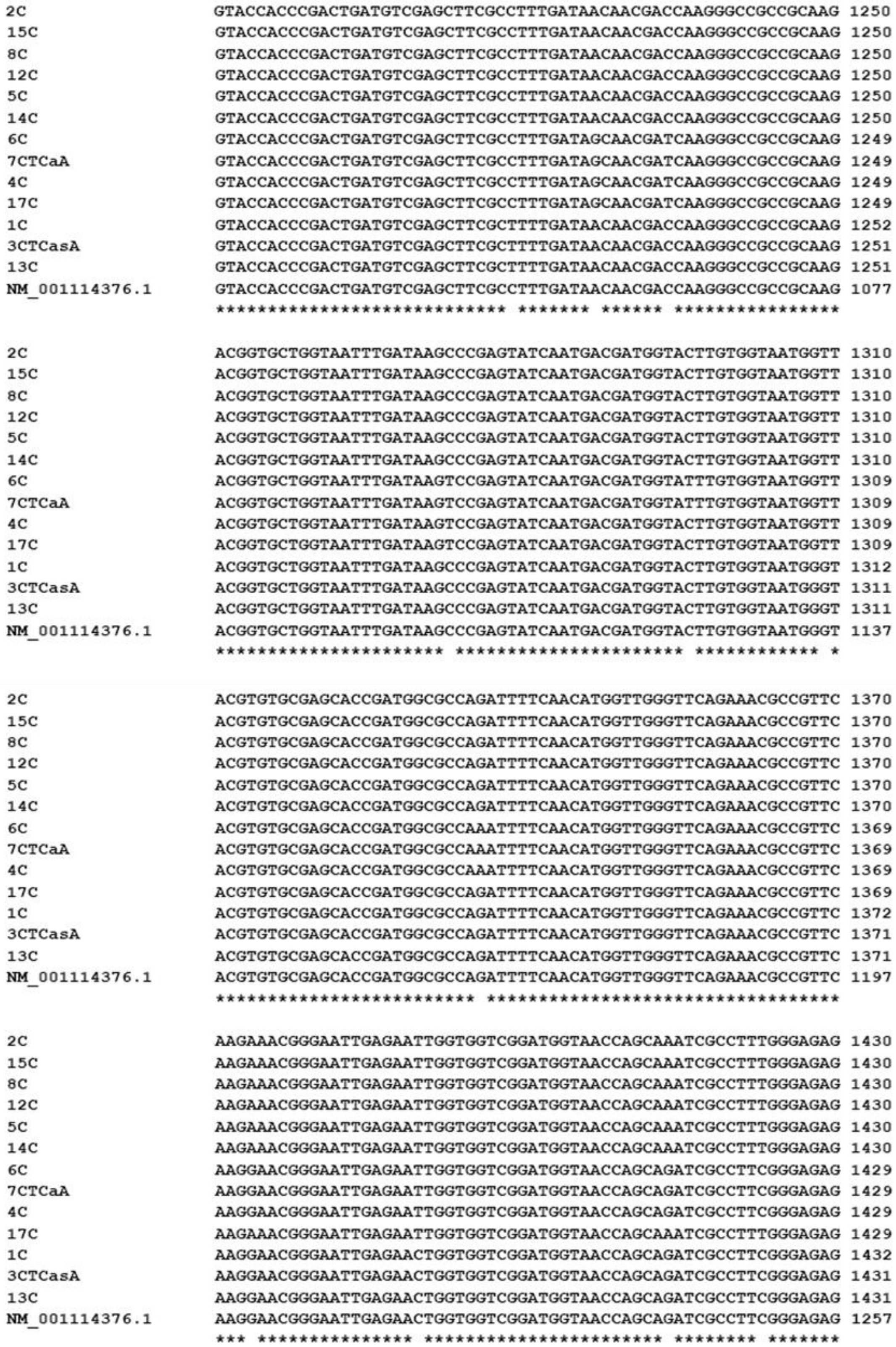
Figure 20 (Cont.)



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12C                 GTACCACCCGACTGATGTCGAGCTTCGCCTTTGATAACAACGACCAAGGGCCGCCGCAAG 1250
5C                  GTACCACCCGACTGATGTCGAGCTTCGCCTTTGATAACAACGACCAAGGGCCGCCGCAAG 1250
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6C                  GTACCACCCGACTGATGTCGAGCTTCGCCTTTGATAGCAACGATCAAGGGCCGCCGCAAG 1249
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4C                  GTACCACCCGACTGATGTCGAGCTTCGCCTTTGATAGCAACGATCAAGGGCCGCCGCAAG 1249
17C                 GTACCACCCGACTGATGTCGAGCTTCGCCTTTGATAGCAACGATCAAGGGCCGCCGCAAG 1249
1C                  GTACCACCCGACTGATGTCGAGCTTCGCTTTTGATAACAACGACCAAGGGCCGCCGCAAG 1252
3CTCasA             GTACCACCCGACTGATGTCGAGCTTCGCTTTTGATAACAACGACCAAGGGCCGCCGCAAG 1251
13C                 GTACCACCCGACTGATGTCGAGCTTCGCTTTTGATAACAACGACCAAGGGCCGCCGCAAG 1251
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5C                  ACGTGTGCGAGCACCGATGGCGCCAGATTTTCAACATGGTTGGGTTCAGAAACGCCGTTC 1370
14C                 ACGTGTGCGAGCACCGATGGCGCCAGATTTTCAACATGGTTGGGTTCAGAAACGCCGTTC 1370
6C                  ACGTGTGCGAGCACCGATGGCGCCAAATTTTCAACATGGTTGGGTTCAGAAACGCCGTTC 1369
7CTCaA              ACGTGTGCGAGCACCGATGGCGCCAAATTTTCAACATGGTTGGGTTCAGAAACGCCGTTC 1369
4C                  ACGTGTGCGAGCACCGATGGCGCCAAATTTTCAACATGGTTGGGTTCAGAAACGCCGTTC 1369
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8C                  AAGAAACGGGAATTGAGAATTGGTGGTCGGATGGTAACCAGCAAATCGCCTTTGGGAGAG 1430
12C                 AAGAAACGGGAATTGAGAATTGGTGGTCGGATGGTAACCAGCAAATCGCCTTTGGGAGAG 1430
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14C                 AAGAAACGGGAATTGAGAATTGGTGGTCGGATGGTAACCAGCAAATCGCCTTTGGGAGAG 1430
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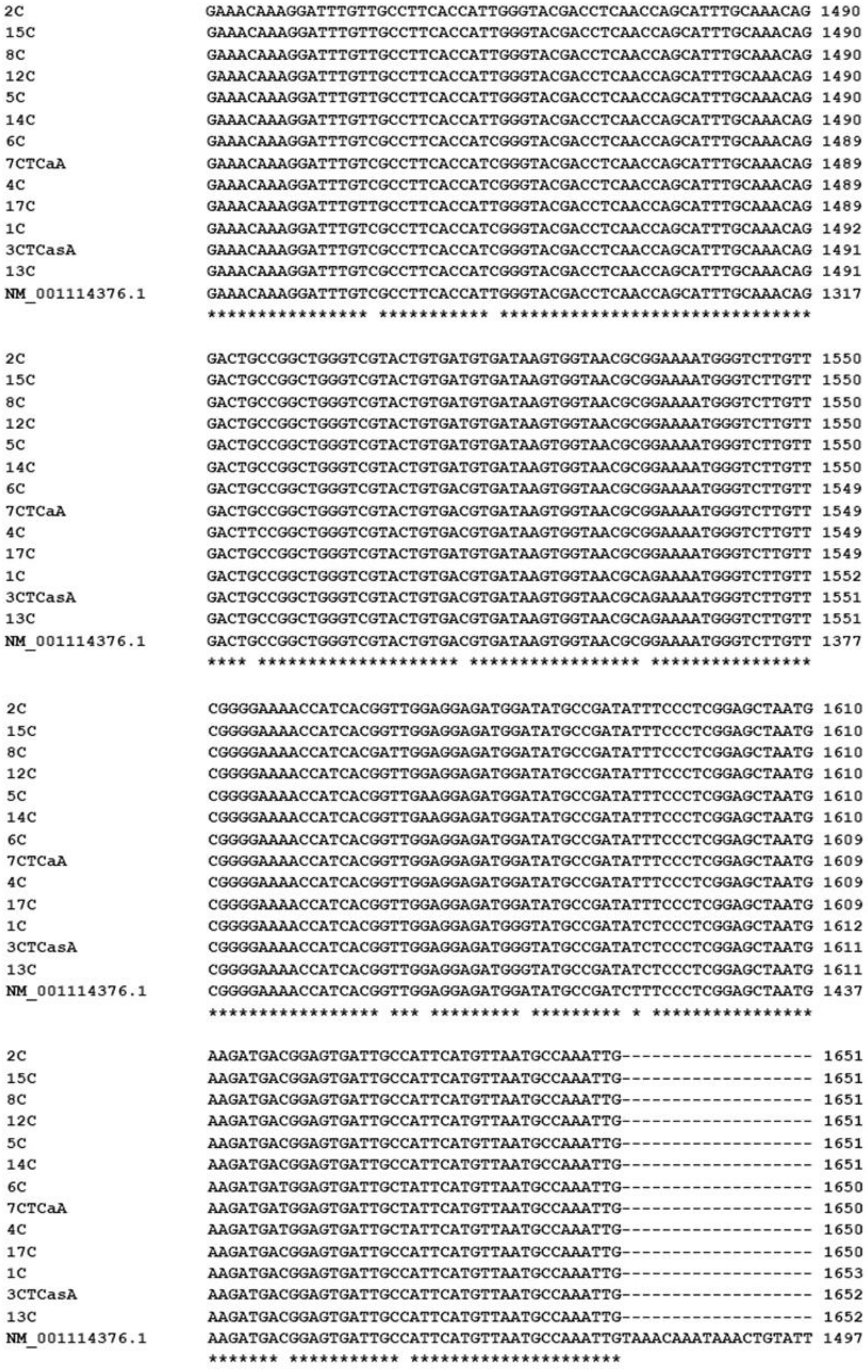
Figure 20 (Cont.)



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8C                  GAAACAAAGGATTTGTTGCCTTCACCATTGGGTACGACCTCAACCAGCATTTGCAAACAG 1490
12C                 GAAACAAAGGATTTGTTGCCTTCACCATTGGGTACGACCTCAACCAGCATTTGCAAACAG 1490
5C                  GAAACAAAGGATTTGTTGCCTTCACCATTGGGTACGACCTCAACCAGCATTTGCAAACAG 1490
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1C                  GAAACAAAGGATTTGTCGCCTTCACCATCGGGTACGACCTCAACCAGCATTTGCAAACAG 1492
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13C                 GAAACAAAGGATTTGTCGCCTTCACCATCGGGTACGACCTCAACCAGCATTTGCAAACAG 1491
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8C                  CGGGGAAAACCATCACGATTGGAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1610
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5C                  CGGGGAAAACCATCACGGTTGAAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1610
14C                 CGGGGAAAACCATCACGGTTGAAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1610
6C                  CGGGGAAAACCATCACGGTTGGAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1609
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4C                  CGGGGAAAACCATCACGGTTGGAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1609
17C                 CGGGGAAAACCATCACGGTTGGAGGAGATGGATATGCCGATATTTCCCTCGGAGCTAATG 1609
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8C                  AAGATGACGGAGTGATTGCCATTCATGTTAATGCCAAATTG------------------- 1651
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5C                  AAGATGACGGAGTGATTGCCATTCATGTTAATGCCAAATTG------------------- 1651
14C                 AAGATGACGGAGTGATTGCCATTCATGTTAATGCCAAATTG------------------- 1651
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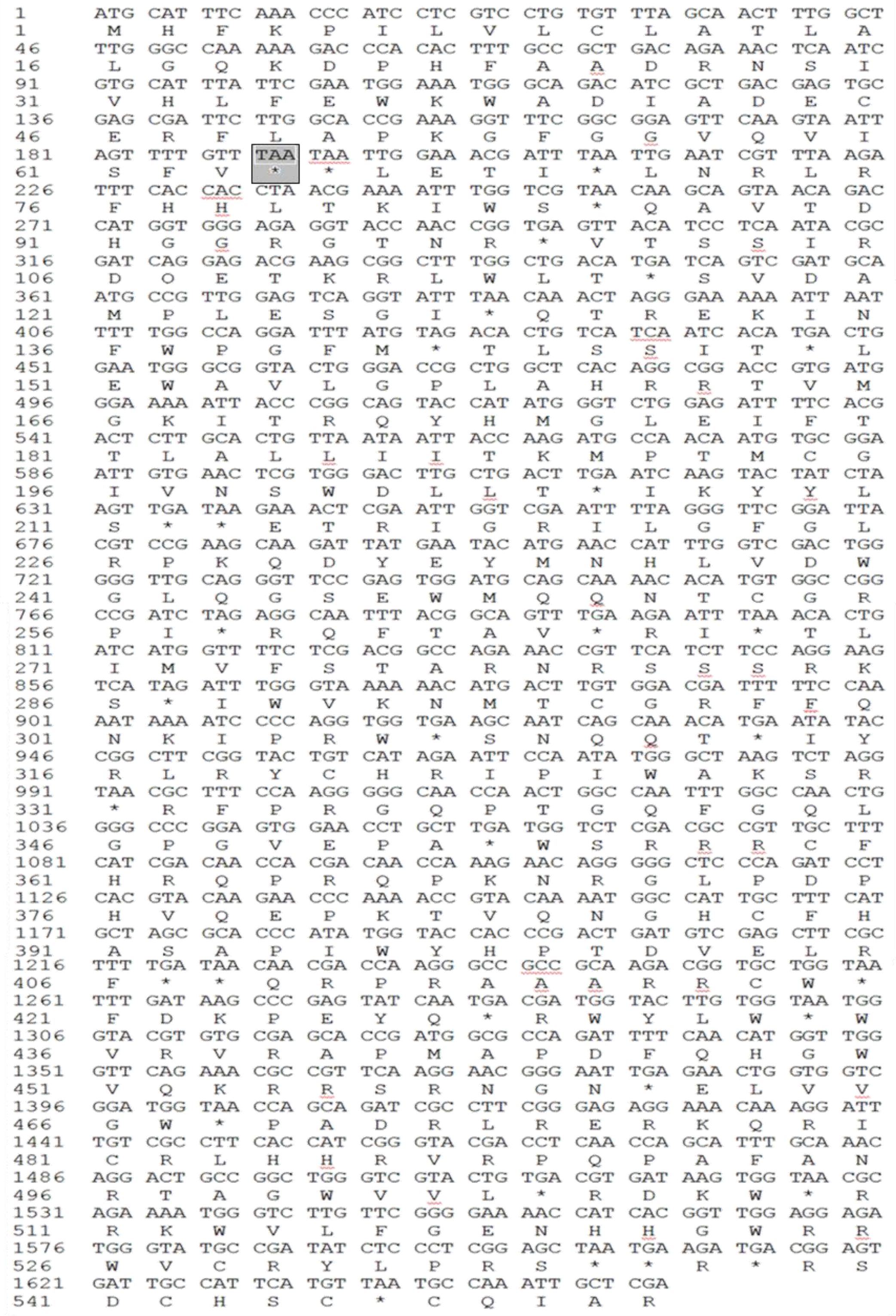
Figure 20 (Cont.)



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46     TTG GGC CAA AAA GAC CCA CAC TTT GCC GCT GAC AGA AAC TCA ATC
16      L   G   Q   K   D   P   H   F   A   A   D   R   N   S   I
91     GTG CAT TTA TTC GAA TGG AAA TGG GCA GAC ATC GCT GAC GAG TGC
31      V   H   L   F   E   W   K   W   A   D   I   A   D   E   C
136    GAG CGA TTC TTG GCA CCG AAA GGT TTC GGC GGA GTT CAA GTA ATT
46      E   R   F   L   A   P   K   G   F   G   G   V   Q   V   I
181    AGT TTT GTT TAA TAA TTG GAA ACG ATT TAA TTG AAT CGT TTA AGA
61      S   F   V   *   *   L   E   T   I   *   L   N   R   L   R
226    TTT CAC CAC CTA ACG AAA ATT TGG TCG TAA CAA GCA GTA ACA GAC
76      F   H   H   L   T   K   I   W   S   *   Q   A   V   T   D
271    CAT GGT GGG AGA GGT ACC AAC CGG TGA GTT ACA TCC TCA ATA CGC
91      H   G   G   R   G   T   N   R   *   V   T   S   S   I   R
316    GAT CAG GAG ACG AAG CGG CTT TGG CTG ACA TGA TCA GTC GAT GCA
106     D   O   E   T   K   R   L   W   L   T   *   S   V   D   A
361    ATG CCG TTG GAG TCA GGT ATT TAA CAA ACT AGG GAA AAA ATT AAT
121     M   P   L   E   S   G   I   *   Q   T   R   E   K   I   N
406    TTT TGG CCA GGA TTT ATG TAG ACA CTG TCA TCA ATC ACA TGA CTG
136     F   W   P   G   F   M   *   T   L   S   S   I   T   *   L
451    GAA TGG GCG GTA CTG GGA CCG CTG GCT CAC AGG CGG ACC GTG ATG
151     E   W   A   V   L   G   P   L   A   H   R   R   T   V   M
496    GGA AAA ATT ACC CGG CAG TAC CAT ATG GGT CTG GAG ATT TTC ACG
166     G   K   I   T   R   Q   Y   H   M   G   L   E   I   F   T
541    ACT CTT GCA CTG TTA ATA ATT ACC AAG ATG CCA ACA ATG TGC GGA
181     T   L   A   L   L   I   I   T   K   M   P   T   M   C   G
586    ATT GTG AAC TCG TGG GAC TTG CTG ACT TGA ATC AAG TAC TAT CTA
196     I   V   N   S   W   D   L   L   T   *   I   K   Y   Y   L
631    AGT TGA TAA GAA ACT CGA ATT GGT CGA ATT TTA GGG TTC GGA TTA
211     S   *   *   E   T   R   I   G   R   I   L   G   F   G   L
676    CGT CCG AAG CAA GAT TAT GAA TAC ATG AAC CAT TTG GTC GAC TGG
226     R   P   K   Q   D   Y   E   Y   M   N   H   L   V   D   W
721    GGG TTG CAG GGT TCC GAG TGG ATG CAG CAA AAC ACA TGT GGC CGG
241     G   L   Q   G   S   E   W   M   Q   Q   N   T   C   G   R
766    CCG ATC TAG AGG CAA TTT ACG GCA GTT TGA AGA ATT TAA ACA CTG
256     P   I   *   R   Q   F   T   A   V   *   R   I   *   T   L
811    ATC ATG GTT TTC TCG ACG GCC AGA AAC CGT TCA TCT TCC AGG AAG
271     I   M   V   F   S   T   A   R   N   R   S   S   S   R   K
856    TCA TAG ATT TGG GTA AAA AAC ATG ACT TGT GGA CGA TTT TTC CAA
286     S   *   I   W   V   K   N   M   T   C   G   R   F   F   Q
901    AAT AAA ATC CCC AGG TGG TGA AGC AAT CAG CAA ACA TGA ATA TAC
301     N   K   I   P   R   W   *   S   N   Q   Q   T   *   I   Y
946    CGG CTT CGG TAC TGT CAT AGA ATT CCA ATA TGG GCT AAG TCT AGG
316     R   L   R   Y   C   H   R   I   P   I   W   A   K   S   R
991    TAA CGC TTT CCA AGG GGG CAA CCA ACT GGC CAA TTT GGC CAA CTG
331     *   R   F   P   R   G   Q   P   T   G   Q   F   G   Q   L
1036   GGG CCC GGA GTG GAA CCT GCT TGA TGG TCT CGA CGC CGT TGC TTT
346     G   P   G   V   E   P   A   *   W   S   R   R   R   C   F
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361     H   R   Q   P   R   Q   P   K   N   R   G   L   P   D   P
1126   CAC GTA CAA GAA CCC AAA ACC GTA CAA AAT GGC CAT TGC TTT CAT
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1171   GCT AGC GCA CCC ATA TGG TAC CAC CCG ACT GAT GTC GAG CTT CGC
391     A   S   A   P   I   W   Y   H   P   T   D   V   E   L   R
1216   TTT TGA TAA CAA CGA CCA AGG GCC GCC GCA AGA CGG TGC TGG TAA
406     F   *   *   Q   R   P   R   A   A   A   R   R   C   W   *
1261   TTT GAT AAG CCC GAG TAT CAA TGA CGA TGG TAC TTG TGG TAA TGG
421     F   D   K   P   E   Y   Q   *   R   W   Y   L   W   *   W
1306   GTA CGT GTG CGA GCA CCG ATG GCG CCA GAT TTT CAA CAT GGT TGG
436     V   R   V   R   A   P   M   A   P   D   F   Q   H   G   W
1351   GTT CAG AAA CGC CGT TCA AGG AAC GGG AAT TGA GAA CTG GTG GTC
451     V   Q   K   R   R   S   R   N   G   N   *   E   L   V   V
1396   GGA TGG TAA CCA GCA GAT CGC CTT CGG GAG AGG AAA CAA AGG ATT
466     G   W   *   P   A   D   R   L   R   E   R   K   Q   R   I
1441   TGT CGC CTT CAC CAT CGG GTA CGA CCT CAA CCA GCA TTT GCA AAC
481     C   R   L   H   H   R   V   R   P   Q   P   A   F   A   N
1486   AGG ACT GCC GGC TGG GTC GTA CTG TGA CGT GAT AAG TGG TAA CGC
496     R   T   A   G   W   V   V   L   *   R   D   K   W   *   R
1531   AGA AAA TGG GTC TTG TTC GGG GAA AAC CAT CAC GGT TGG AGG AGA
511     R   K   W   V   L   F   G   E   N   H   H   G   W   R   R
1576   TGG GTA TGC CGA TAT CTC CCT CGG AGC TAA TGA AGA TGA CGG AGT
526     W   V   C   R   Y   L   P   R   S   *   *   R   *   R   S
1621   GAT TGC CAT TCA TGT TAA TGC CAA ATT GCT CGA
541     D   C   H   S   C   *   C   Q   I   A   R
```

Figure 21 **The cDNA sequence and its deduced protein sequence of *Tribolium castaneum* α-amylase gene. Abnormal stop codon (grey box) is shown in the first extra region**

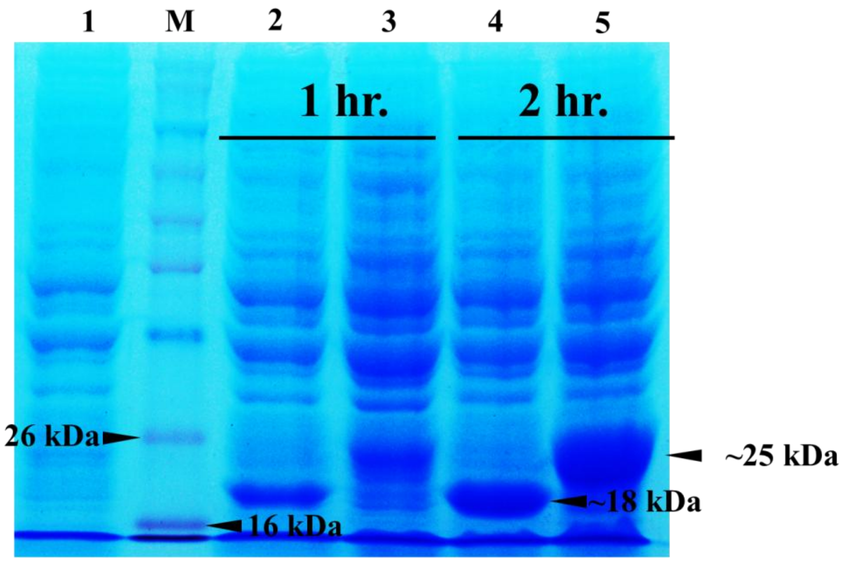


Figure 22 10% SDS-PAGE gel of 1650 bp α-amylase - pET32a (+) recombinant cells was induced with 1mM IPTG at 37 °C. Lane M: protein ladder, lane 1: non induced cells, lane 2, 4: pET32a (+) no insert cells induced for 1 and 2 hr, lane 3, 5: 1650 bp α-amylase - pET32a (+) recombinant cells induced for 1 and 2 hr, respectively

Growth, survival and α-amylase gene expression of *Tribolium castaneum* on different starch diets

Carbohydrate is an essential energy-producing nutrient required for both optimal larval growth and adult longevity for the majority of insects (Dadd, 1985), including *Tribolium castaneum*, one of the major insect pests in Thai agricultural. Generally, *Tribolium castaneum* is capable of dispersal in various carbohydrate foods, including grains and flour stored in a storage area (Hagstrum, 1973). However, the oviposition rate and emergence of new adults depend greatly on the quality and type of the substrate available (Ziegler, 1976). In previous studies, the preference of stored food products for *Tribolium* sp. growth and development were identified (Verner, 1971). The different types of starch contain different nutrients that can considerably affect *Tribolium* sp. development (Campbell and Runnion, 2003).

Our results showed that the growth of 3 week old *Tribolium castaneum* larvae reared in wheat flour supplemented with 5% of yeast powder was greater than the one reared in KDML 105 rice powder (Figure 23). According to Wong and Lee (2011), the proximate nutrition component analysis showed that among the diets tested, wheat flour contains higher protein, but less carbohydrate than rice. Therefore, our results were concordant with the Wong and Lee (2011), Wool and Noiman (1980), (Sokoloff, 1974) reports, which showed that the development of *Tribolium castaneum* was influenced by the availability of protein in the flour. On the other hand, rice powder which contains higher amounts of carbohydrate, but less amount of protein, reduced the growth and development of *Tribolium castaneum.* The results implied that the different types of starch influence growth and developmental rate of *Tribolium castaneum* in different ways. When the protein content in the diet was high, more adult beetles emerged. In contrast, fewer adults developed in diets with high carbohydrate content. Therefore, the adult beetles reared in KDML 105 rice powder possess the higher mortality rate than the beetle reared in wheat flour supplemented with 5% of yeast powder (Figure 24).

The mRNA expression was detected by RT-PCR from the reared insects at the age of 1, 2, 3, 4, 6 and 8 weeks. The 430 bp of insect β-actin gene were amplified in every week (Figure 25A). In contrast, the 1,470 bp of TcAm gene were amplified in 1 and 2 week old insects reared in both diets. However, there was no 1,470 TcAm detected in 3 week old insects reared in wheat supplemented with 5% of yeast powder. Alternatively, the 1,650 bp of TcAm gene appeared instead of 1,470 bp TcAm in the insects reared in wheat flour supplemented with 5% of yeast powder, and were detected in 3, 4, 6 and 8 week old insects reared in both diets (Figure 25B).

Since the expression of 1,650 bp TcAm gene presented that their protein possess no amylolytic activity, the 1,470 bp TcAm gene was supposed to be the active α-amylase gene which exhibited the amylase activity. The result indicated that the *Tribolium castaneum* α-amylase activity (from 1,470 bp TcAm) may decrease in 3, 4, 6 and 8 week old insects. Acording to Wool and Noiman (1980) reported the amylase activity of the confused flour beetle, *Tribolium confusum* reared on flour with brewer’s yeast varied depending on age. The amylase activity increased with larval age up to 16 days, and was stable between 16 and 22 days (2-3 weeks).

Hypothetically, when both diets have been replaced with the fresh ones, high nutrition especially carbohydrate in the fresh diets could induce the adult *Tribolium castaneum* to adapt itself for survival in the early period (about 16 day) by expressing the α-amylase gene. Subsequently, the adult insects would decrease the α-amylase geneexpression, but partially remained for survival and propagation. Moreover, the KDML 105 contains less protein, but higher carbohydrate than wheat flour supplemented with 5% yeast diet. Therefore, this could induce higher α-amylase gene expression in the insects reared in KDML 105, especially, in week 3. Furthermore, the results obtained were concordant to the previous studies, which indicated that the expression of α-amylase gene correlates to the amount of carbohydrate in the diet. However, the mechanism of this correlation is still unknown.

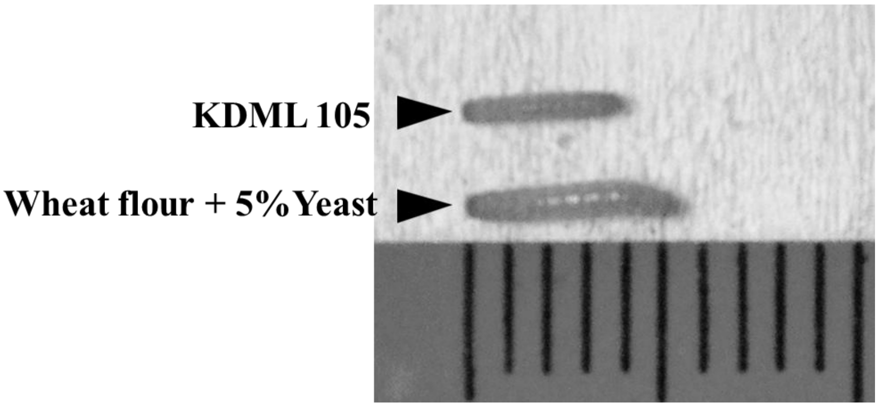


Figure 23 Larvae of *Tribolium castaneum* were fed with difference diet after 3 week

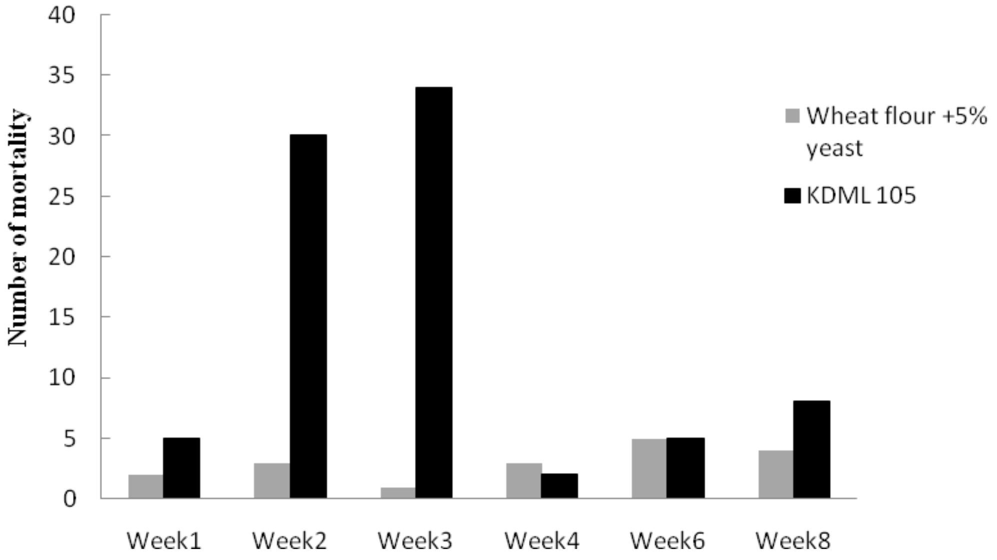


Figure 24 Effect of different diets on *Tribolium castaneum* mortality

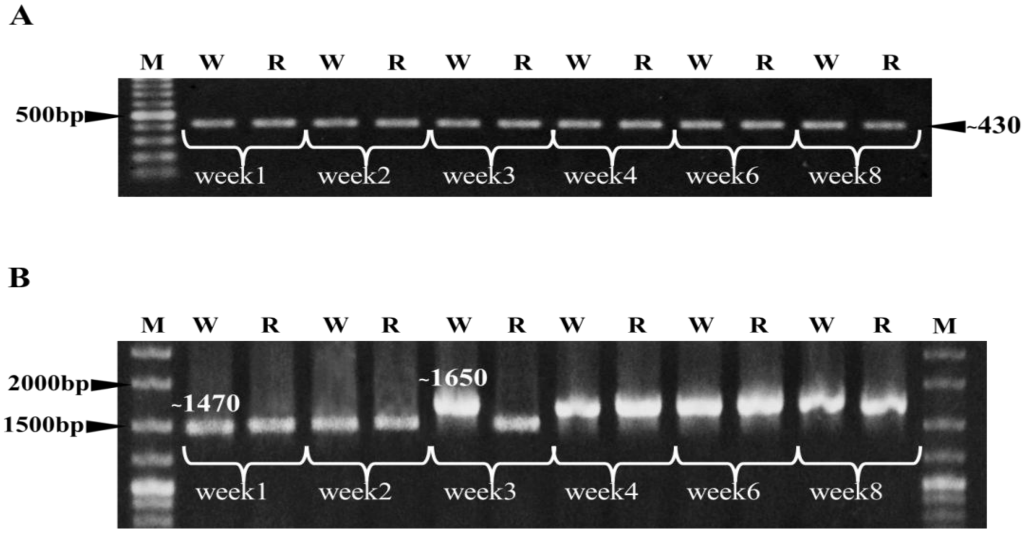


Figure 25 Agarose gel electrophoresis of PCR product amplified from *Tribolium castaneum* cDNA, isolated RNA in 1, 2, 3, 4, 6 and 8 week. W: Wheat flour + 5% yeast powder, R: Jasmine rice KDML 105, (A) M: 100 bp DNA molecular weight ladder, Lane 1-12: insect β-actin genes were amplified in each week. (B) M: 100 bp DNA molecular weight ladder, Lane 1-12: *Tribolium castaneum* α-amylase genes were amplified in each week

Cloning and sequence analysis of 1,470 bp TcAm gene

The 1,470 bp of α-amylase cDNAs were isolated from agarose gel prior to being cloned by TA-cloning system and transformed into *E.coli* (DH5α). Three recombinant clones were obtained prior to the screening with colony PCR and *BamHI* and *XhoI* restriction enzyme analysis (Figure 26 and Figure 27). Subsequent to the isolation of plasmid, the nucleotide sequences were determined.

Each the 1,470 bp cDNA nucleotide sequences of the three TcAm clones obtained, which start with ATG codon and end with TTG translational stop codon (GenBank accession no. KF247314, KF247315, KF247316), encoded 490 amino acids with a putative 17 amino acid signal peptide. The predicted molecular mass protein of TcAm was 53.3 kDa and the estimated isoelectric point (p*I*) was 4.92. The clustalw alignment of TcAm nucleotide sequences and amino acids sequences with *T.castaneum* α-amylase NCBI database were shown in Figure 28 and Figure 29.

According to the sequence alignment of TcAm and the NCBI database (Figure 29), the three active site residues of TcAm perfectly matched with the corresponding residues (Asp204, Glu 241 and Asp303) of NCBI database, as well as the residues creating the calcium (Asn117, Arg165, Asp174, and His218) and chloride (Arg202, Asn304, Arg340) binding sites of TcAm. The α-amylase molecule is highly conserved among various organisms and other insects, demonstrating conserved active site residues, calcium binding and chloride binding site residues.

The comparison between the three clones of TcAM amino acid sequences and other α-amylase amino acid sequences, which were PPA: Asp197, Glu233 and Asp300, (Brayer, et al., 1995), TMA: Asp185, Glu222 and Asp287, (Strobl, et al., 1998), and *Sitophilus oryzae* α-amylase: Asp 202, Glu 238, Asp 303, (Lertkaeo, 2011), showed that the catalytic residues are located in similar amino acid, a glutamic acid residue and two aspartic acid residues, but at different positions.

Furthermore, the TcAMobtained in this study was found to possess the chloride dependent amino acids similar to other α-amylases, such as Arg183, Asn285 and Arg321 in TMA, Arg200, Asn304 and Arg337 in *Sitophilus oryzae*, and Arg210, Asn313 and Arg352 in disk abalone (*Haliotis discus discus*) (Chamilani Nikapitiya, et al., 2009), which are strictly conserved in chloride-dependent α-amylases. Similarly, the TcAM calcium binding residues were also found to be conserved with other insects such as, Asn98, Arg146, Asp155, and His189 in TMA and Asn 115, Arg162, Asp171 and His206 in *Sitophilus oryzae*.

Expression & Purification of recombinant *Tribolium castaneum* α-amylase

The pET32a(+) – 1,470 bp TcAmrecombinant protein was successfully produced in *E. coli*BL21(DE3)pLysS at 37 °C, 1 hr by the induction with1 mM IPTG under the control of T7 RNA polymerase promoter 6x His-tagged. However, the pET32a(+) – 1,470 bp TcAmwas initially expressed as 6xHis-tagged and the others fusion protein from pET32a(+).Figure 30 shows SDS-PAGE analysis of the total protein from whole-cell extracts of non-induce compared with IPTG-induced cell cultures. In induced cells, an intense band of about 72 kDa can easily see. The result was correlated with expected size of the fusion protein-TcAm 72 kDa that predicted by ExPASy Compute pI/Mw tool (53.3 kDa from TcAm plus 18.7 kDa from pET32a(+)Thioredoxin/Hitidine tag from vector).

The expression vectors employed in this study are capable of producing fusion proteins, which are cleavable at the (His) 6-tagged protein sequences for the purification. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, Ni^{2+} ion, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal (Joshua A. Bornhorst and Joseph J. Falke, 2000). The expressed recombinant protein was efficiently purified with one-step purification procedure using Ni^{2+} affinity chromatography Columns, which were loaded with 2 ml of sample and washed with binding buffer (20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 20 mM imidazole) prior to the elution. Subsequent to the washing of the matrix material, peptides containing polyhistidine sequences were eluted by adding free imidazole to the column buffer. The histidine analog imidazole could also be used to competitively elute the bound polyhistidine residues. Proteins were eluted using gradient from 0 to 100% elution buffer (20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 0.5 M imidazole), followed by completely eluted with 100% elution buffer.

SDS-PAGE was carried out to determine the purification and expression level of TcAm. SDS-PAGE analysis indicated that the enzyme was efficiently purified and a single band corresponding to approximately 72 kDa was obtained (Figure 31).

According to our study, the insect α-amylases have been studied in several insect. Numerous reports showed that molecular weight of insect α-amylases are approximately 51-55 kDa in length such as *Sitophilus oryzae* α-amylase: 54 kDa (Lertkaeo, 2011), *Rhyzopertha dominica* α-amylase: 52 kDa (Smriti Priya, 2010) and *Tenebrio molitor* : 51.3 kDa (Strobl, 1998). The obtained protein was identified by LC-MS/MS. After induction, the protein band about 72 kDa was excised from SDS-PAGE, trypsin digested and analyzed by LC-MS/MS. Mascot search of MS/MS of protein band showed the matched peptide to the *Tribolium castaneum* α-amylase (Figure 32 and Figure 33).

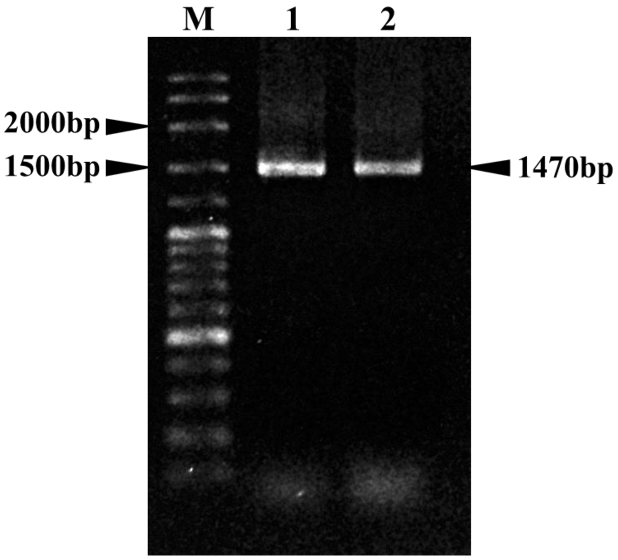


Figure 26 Agarose gel electrophoresis of colony PCR screening of the 1,470 bp TcAm recombinant plasmid with specific primers, M: 100 bp DNA molecular weight Ladder, Lane 1-2 PCR product of 1,650 bp TcAm

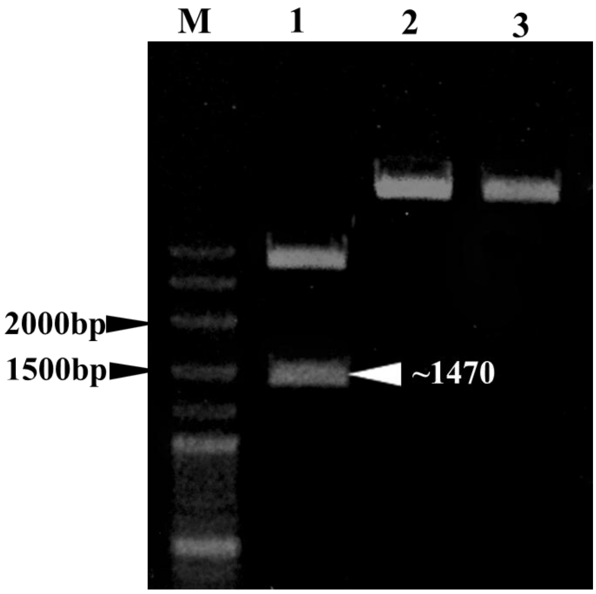
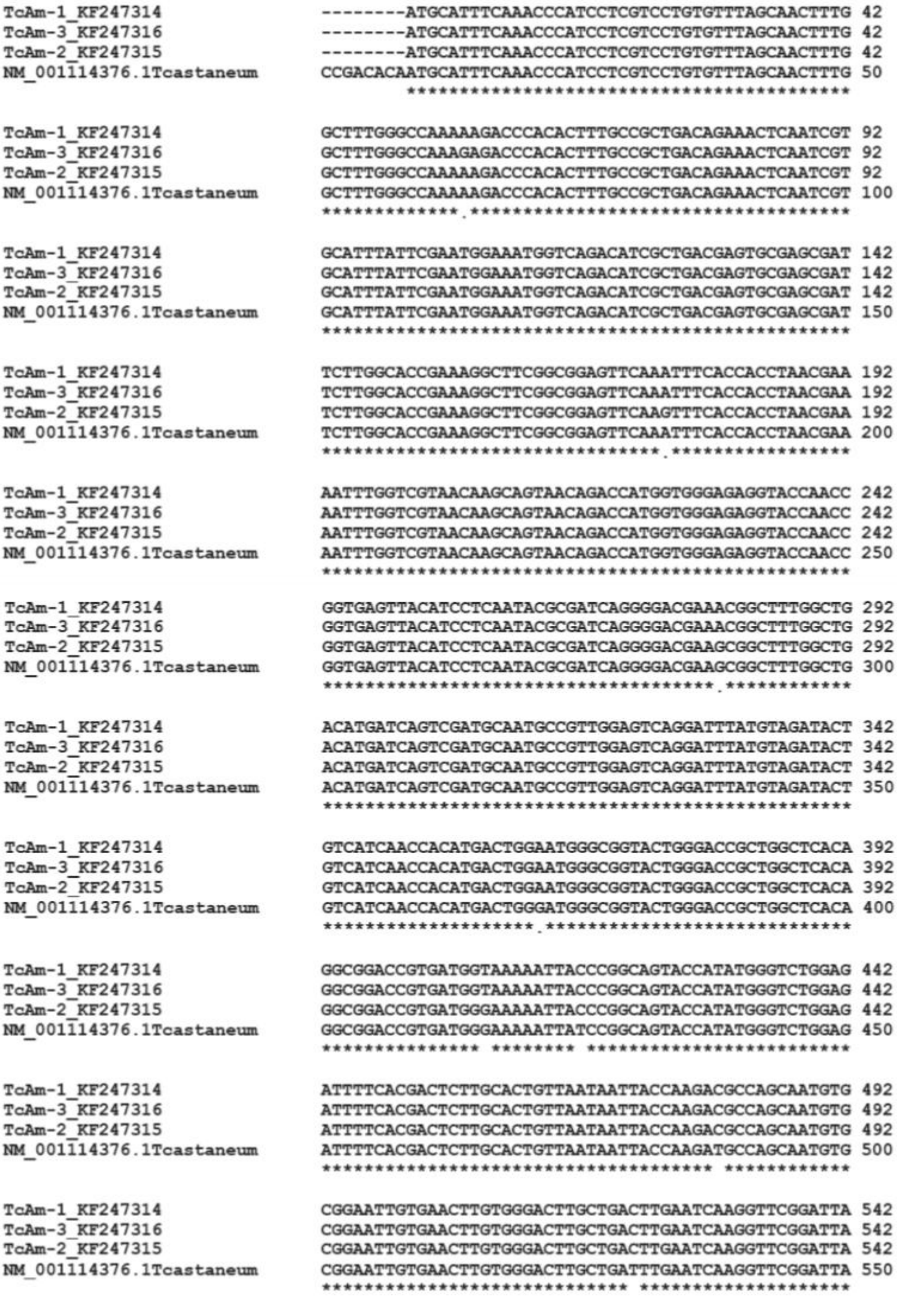


Figure 27 **Agarose gel electrophoresis of BamHI and XhoI restriction enzyme analysis of 1,470 bp TcAM , M: 100 bp DNA molecular weight Ladder, Lane 1: Recombinant plasmid digested with *BamHI* and *XhoI*, Lane 2, Recombinant plasmid digested with *BamHI*, Lane 3: Recombinant plasmid digested with *XhoI***



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TcAm-2_KF247315                     --------ATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTG 42
NM_001114376.1Tcastaneum            CCGACACAATGCATTTCAAACCCATCCTCGTCCTGTGTTTAGCAACTTTG 50
                                            ******************************************

TcAm-1_KF247314                     GCTTTGGGCCAAAAAGACCCACACTTTGCCGCTGACAGAAACTCAATCGT 92
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TcAm-2_KF247315                     GCTTTGGGCCAAAAAGACCCACACTTTGCCGCTGACAGAAACTCAATCGT 92
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                                    *************.************************************

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TcAm-2_KF247315                     GCATTTATTCGAATGGAAATGGTCAGACATCGCTGACGAGTGCGAGCGAT 142
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TcAm-3_KF247316                     TCTTGGCACCGAAAGGCTTCGGCGGAGTTCAAATTTCACCACCTAACGAA 192
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                                    **************************************************

TcAm-1_KF247314                     GGTGAGTTACATCCTCAATACGCGATCAGGGGACGAAACGGCTTTGGCTG 292
TcAm-3_KF247316                     GGTGAGTTACATCCTCAATACGCGATCAGGGGACGAAACGGCTTTGGCTG 292
TcAm-2_KF247315                     GGTGAGTTACATCCTCAATACGCGATCAGGGGACGAAGCGGCTTTGGCTG 292
NM_001114376.1Tcastaneum            GGTGAGTTACATCCTCAATACGCGATCAGGGGACGAAGCGGCTTTGGCTG 300
                                    *************************************.************

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TcAm-3_KF247316                     ACATGATCAGTCGATGCAATGCCGTTGGAGTCAGGATTTATGTAGATACT 342
TcAm-2_KF247315                     ACATGATCAGTCGATGCAATGCCGTTGGAGTCAGGATTTATGTAGATACT 342
NM_001114376.1Tcastaneum            ACATGATCAGTCGATGCAATGCCGTTGGAGTCAGGATTTATGTAGATACT 350
                                    **************************************************

TcAm-1_KF247314                     GTCATCAACCACATGACTGGAATGGGCGGTACTGGGACCGCTGGCTCACA 392
TcAm-3_KF247316                     GTCATCAACCACATGACTGGAATGGGCGGTACTGGGACCGCTGGCTCACA 392
TcAm-2_KF247315                     GTCATCAACCACATGACTGGAATGGGCGGTACTGGGACCGCTGGCTCACA 392
NM_001114376.1Tcastaneum            GTCATCAACCACATGACTGGGATGGGCGGTACTGGGACCGCTGGCTCACA 400
                                    ********************.*****************************

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TcAm-2_KF247315                     GGCGGACCGTGATGGGAAAAATTACCCGGCAGTACCATATGGGTCTGGAG 442
NM_001114376.1Tcastaneum            GGCGGACCGTGATGGGAAAAATTATCCGGCAGTACCATATGGGTCTGGAG 450
                                    *************** ******** *************************

TcAm-1_KF247314                     ATTTTCACGACTCTTGCACTGTTAATAATTACCAAGACGCCAGCAATGTG 492
TcAm-3_KF247316                     ATTTTCACGACTCTTGCACTGTTAATAATTACCAAGACGCCAGCAATGTG 492
TcAm-2_KF247315                     ATTTTCACGACTCTTGCACTGTTAATAATTACCAAGACGCCAGCAATGTG 492
NM_001114376.1Tcastaneum            ATTTTCACGACTCTTGCACTGTTAATAATTACCAAGATGCCAGCAATGTG 500
                                    ************************************* ************

TcAm-1_KF247314                     CGGAATTGTGAACTTGTGGGACTTGCTGACTTGAATCAAGGTTCGGATTA 542
TcAm-3_KF247316                     CGGAATTGTGAACTTGTGGGACTTGCTGACTTGAATCAAGGTTCGGATTA 542
TcAm-2_KF247315                     CGGAATTGTGAACTTGTGGGACTTGCTGACTTGAATCAAGGTTCGGATTA 542
NM_001114376.1Tcastaneum            CGGAATTGTGAACTTGTGGGACTTGCTGATTTGAATCAAGGTTCGGATTA 550
                                    ***************************** ********************
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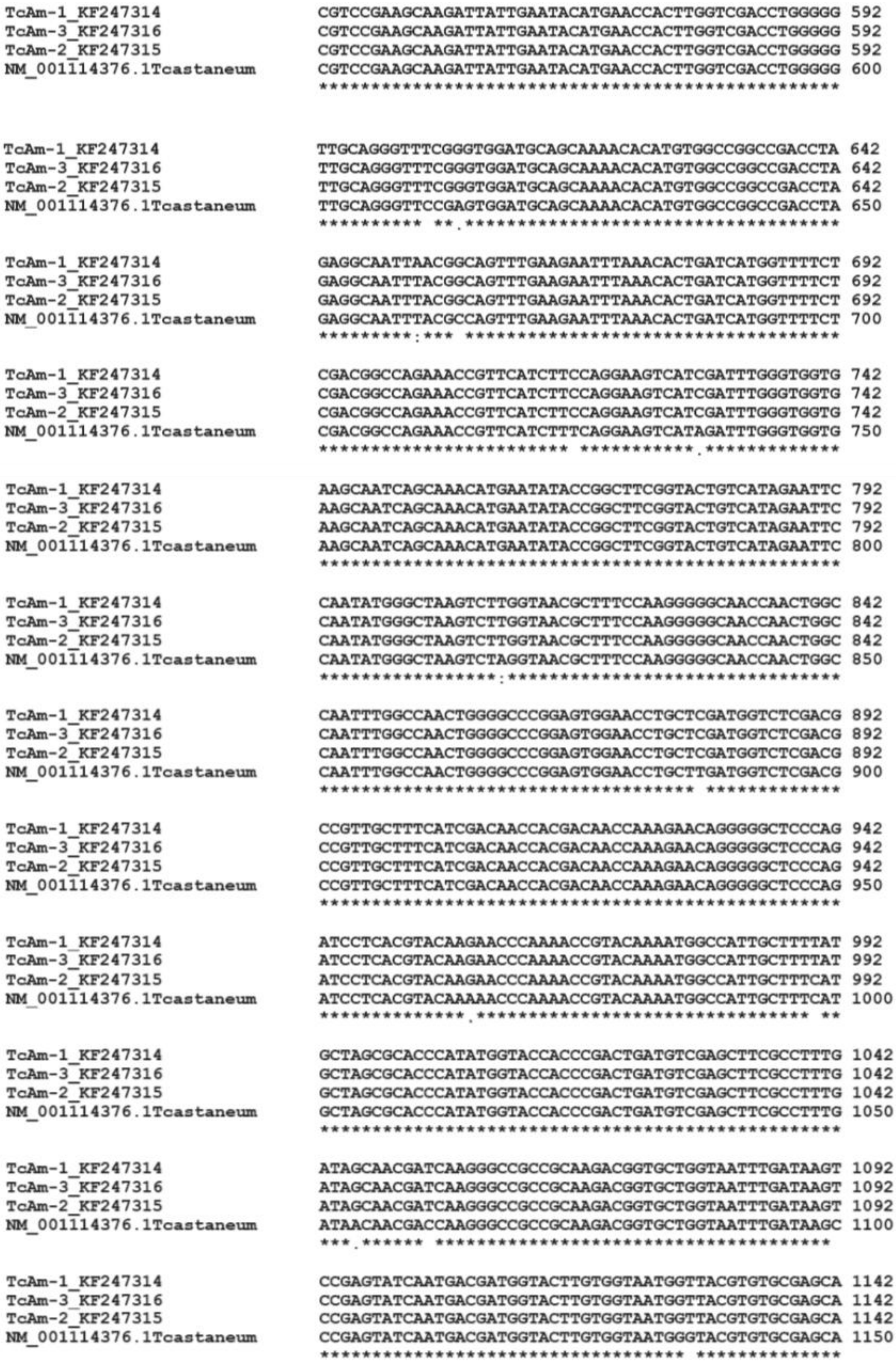
Figure 28 Three clones of 1,470 bp *Tribolium castaneum* α-amylase genes nucleotide sequence alignment compared with the NCBI nucleotide database (NCBI Reference Sequence: NM_001114376.1) tool



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TcAm-3_KF247316             CGTCCGAAGCAAGATTATTGAATACATGAACCACTTGGTCGACCTGGGGG 592
TcAm-2_KF247315             CGTCCGAAGCAAGATTATTGAATACATGAACCACTTGGTCGACCTGGGGG 592
NM_001114376.1Tcastaneum    CGTCCGAAGCAAGATTATTGAATACATGAACCACTTGGTCGACCTGGGGG 600
                            **************************************************


TcAm-1_KF247314             TTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCGACCTA 642
TcAm-3_KF247316             TTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCGACCTA 642
TcAm-2_KF247315             TTGCAGGGTTTCGGGTGGATGCAGCAAAACACATGTGGCCGGCCGACCTA 642
NM_001114376.1Tcastaneum    TTGCAGGGTTCCGAGTGGATGCAGCAAAACACATGTGGCCGGCCGACCTA 650
                            ********** **.************************************

TcAm-1_KF247314             GAGGCAATTAACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCT 692
TcAm-3_KF247316             GAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCT 692
TcAm-2_KF247315             GAGGCAATTTACGGCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCT 692
NM_001114376.1Tcastaneum    GAGGCAATTTACGCCAGTTTGAAGAATTTAAACACTGATCATGGTTTTCT 700
                            *********:*** ************************************

TcAm-1_KF247314             CGACGGCCAGAAACCGTTCATCTTCCAGGAAGTCATCGATTTGGGTGGTG 742
TcAm-3_KF247316             CGACGGCCAGAAACCGTTCATCTTCCAGGAAGTCATCGATTTGGGTGGTG 742
TcAm-2_KF247315             CGACGGCCAGAAACCGTTCATCTTCCAGGAAGTCATCGATTTGGGTGGTG 742
NM_001114376.1Tcastaneum    CGACGGCCAGAAACCGTTCATCTTTCAGGAAGTCATAGATTTGGGTGGTG 750
                            ************************ ***********.*************

TcAm-1_KF247314             AAGCAATCAGCAAACATGAATATACCGGCTTCGGTACTGTCATAGAATTC 792
TcAm-3_KF247316             AAGCAATCAGCAAACATGAATATACCGGCTTCGGTACTGTCATAGAATTC 792
TcAm-2_KF247315             AAGCAATCAGCAAACATGAATATACCGGCTTCGGTACTGTCATAGAATTC 792
NM_001114376.1Tcastaneum    AAGCAATCAGCAAACATGAATATACCGGCTTCGGTACTGTCATAGAATTC 800
                            **************************************************

TcAm-1_KF247314             CAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGGCAACCAACTGGC 842
TcAm-3_KF247316             CAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGGCAACCAACTGGC 842
TcAm-2_KF247315             CAATATGGGCTAAGTCTTGGTAACGCTTTCCAAGGGGGCAACCAACTGGC 842
NM_001114376.1Tcastaneum    CAATATGGGCTAAGTCTAGGTAACGCTTTCCAAGGGGGCAACCAACTGGC 850
                            *****************:********************************

TcAm-1_KF247314             CAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTCGATGGTCTCGACG 892
TcAm-3_KF247316             CAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTCGATGGTCTCGACG 892
TcAm-2_KF247315             CAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTCGATGGTCTCGACG 892
NM_001114376.1Tcastaneum    CAATTTGGCCAACTGGGGCCCGGAGTGGAACCTGCTTGATGGTCTCGACG 900
                            ************************************ *************

TcAm-1_KF247314             CCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAG 942
TcAm-3_KF247316             CCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAG 942
TcAm-2_KF247315             CCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAG 942
NM_001114376.1Tcastaneum    CCGTTGCTTTCATCGACAACCACGACAACCAAAGAACAGGGGGCTCCCAG 950
                            **************************************************

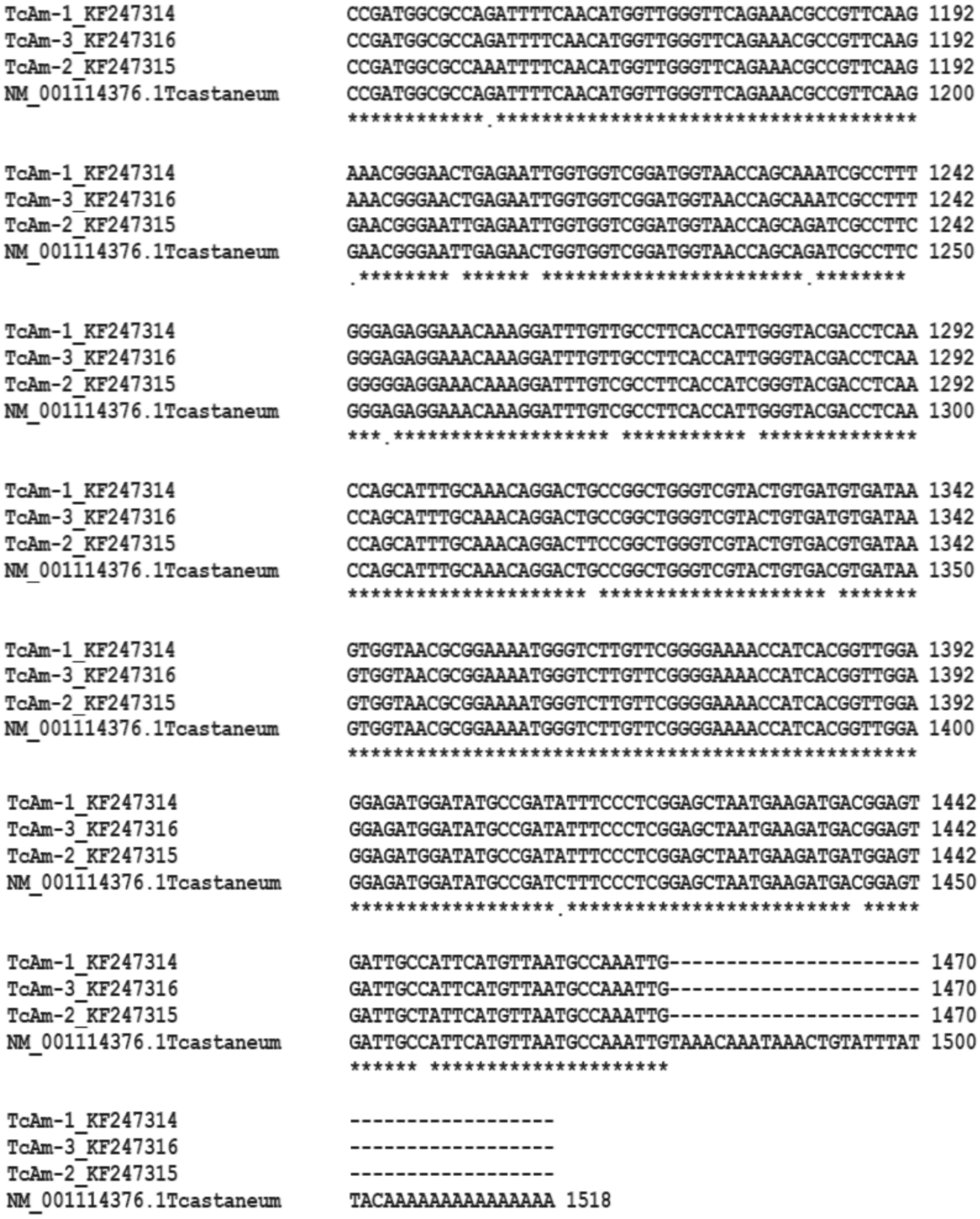
TcAm-1_KF247314             ATCCTCACGTACAAGAACCCAAAACCGTACAAAATGGCCATTGCTTTTAT 992
TcAm-3_KF247316             ATCCTCACGTACAAGAACCCAAAACCGTACAAAATGGCCATTGCTTTTAT 992
TcAm-2_KF247315             ATCCTCACGTACAAGAACCCAAAACCGTACAAAATGGCCATTGCTTTCAT 992
NM_001114376.1Tcastaneum    ATCCTCACGTACAAAAACCCAAAACCGTACAAAATGGCCATTGCTTTCAT 1000
                            **************.******************************** **

TcAm-1_KF247314             GCTAGCGCACCCATATGGTACCACCCGACTGATGTCGAGCTTCGCCTTTG 1042
TcAm-3_KF247316             GCTAGCGCACCCATATGGTACCACCCGACTGATGTCGAGCTTCGCCTTTG 1042
TcAm-2_KF247315             GCTAGCGCACCCATATGGTACCACCCGACTGATGTCGAGCTTCGCCTTTG 1042
NM_001114376.1Tcastaneum    GCTAGCGCACCCATATGGTACCACCCGACTGATGTCGAGCTTCGCCTTTG 1050
                            **************************************************

TcAm-1_KF247314             ATAGCAACGATCAAGGGCCGCCGCAAGACGGTGCTGGTAATTTGATAAGT 1092
TcAm-3_KF247316             ATAGCAACGATCAAGGGCCGCCGCAAGACGGTGCTGGTAATTTGATAAGT 1092
TcAm-2_KF247315             ATAGCAACGATCAAGGGCCGCCGCAAGACGGTGCTGGTAATTTGATAAGT 1092
NM_001114376.1Tcastaneum    ATAACAACGACCAAGGGCCGCCGCAAGACGGTGCTGGTAATTTGATAAGC 1100
                            ***.****** **************************************

TcAm-1_KF247314             CCGAGTATCAATGACGATGGTACTTGTGGTAATGGTTACGTGTGCGAGCA 1142
TcAm-3_KF247316             CCGAGTATCAATGACGATGGTACTTGTGGTAATGGTTACGTGTGCGAGCA 1142
TcAm-2_KF247315             CCGAGTATCAATGACGATGGTACTTGTGGTAATGGTTACGTGTGCGAGCA 1142
NM_001114376.1Tcastaneum    CCGAGTATCAATGACGATGGTACTTGTGGTAATGGGTACGTGTGCGAGCA 1150
                            *********************************** **************
```

Figure 28 (Cont.)



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TcAm-1_KF247314             CCGATGGCGCCAGATTTTCAACATGGTTGGGTTCAGAAACGCCGTTCAAG 1192
TcAm-3_KF247316             CCGATGGCGCCAGATTTTCAACATGGTTGGGTTCAGAAACGCCGTTCAAG 1192
TcAm-2_KF247315             CCGATGGCGCCAAATTTTCAACATGGTTGGGTTCAGAAACGCCGTTCAAG 1192
NM_001114376.1Tcastaneum    CCGATGGCGCCAGATTTTCAACATGGTTGGGTTCAGAAACGCCGTTCAAG 1200
                            ************.*************************************

TcAm-1_KF247314             AAACGGGAACTGAGAATTGGTGGTCGGATGGTAACCAGCAAATCGCCTTT 1242
TcAm-3_KF247316             AAACGGGAACTGAGAATTGGTGGTCGGATGGTAACCAGCAAATCGCCTTT 1242
TcAm-2_KF247315             GAACGGGAATTGAGAATTGGTGGTCGGATGGTAACCAGCAGATCGCCTTC 1242
NM_001114376.1Tcastaneum    GAACGGGAATTGAGAACTGGTGGTCGGATGGTAACCAGCAGATCGCCTTC 1250
                            .******** ****** ***********************.********

TcAm-1_KF247314             GGGAGAGGAAACAAAGGATTTGTTGCCTTCACCATTGGGTACGACCTCAA 1292
TcAm-3_KF247316             GGGAGAGGAAACAAAGGATTTGTTGCCTTCACCATTGGGTACGACCTCAA 1292
TcAm-2_KF247315             GGGGGAGGAAACAAAGGATTTGTCGCCTTCACCATCGGGTACGACCTCAA 1292
NM_001114376.1Tcastaneum    GGGAGAGGAAACAAAGGATTTGTCGCCTTCACCATTGGGTACGACCTCAA 1300
                            ***.******************* *********** **************

TcAm-1_KF247314             CCAGCATTTGCAAACAGGACTGCCGGCTGGGTCGTACTGTGATGTGATAA 1342
TcAm-3_KF247316             CCAGCATTTGCAAACAGGACTGCCGGCTGGGTCGTACTGTGATGTGATAA 1342
TcAm-2_KF247315             CCAGCATTTGCAAACAGGACTTCCGGCTGGGTCGTACTGTGACGTGATAA 1342
NM_001114376.1Tcastaneum    CCAGCATTTGCAAACAGGACTGCCGGCTGGGTCGTACTGTGACGTGATAA 1350
                            ********************* ******************** *******

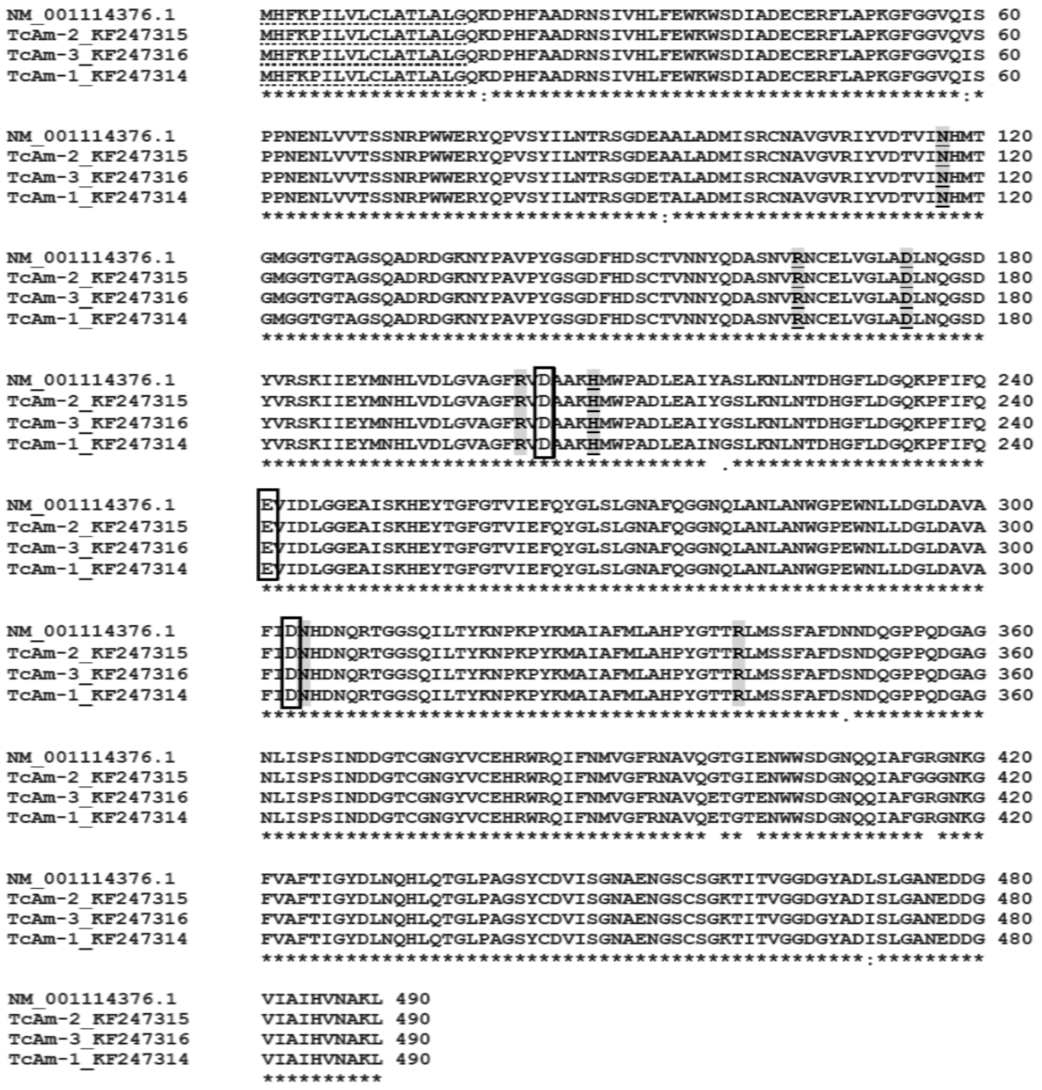
TcAm-1_KF247314             GTGGTAACGCGGAAAATGGGTCTTGTTCGGGGAAAACCATCACGGTTGGA 1392
TcAm-3_KF247316             GTGGTAACGCGGAAAATGGGTCTTGTTCGGGGAAAACCATCACGGTTGGA 1392
TcAm-2_KF247315             GTGGTAACGCGGAAAATGGGTCTTGTTCGGGGAAAACCATCACGGTTGGA 1392
NM_001114376.1Tcastaneum    GTGGTAACGCGGAAAATGGGTCTTGTTCGGGGAAAACCATCACGGTTGGA 1400
                            **************************************************

TcAm-1_KF247314             GGAGATGGATATGCCGATATTTCCCTCGGAGCTAATGAAGATGACGGAGT 1442
TcAm-3_KF247316             GGAGATGGATATGCCGATATTTCCCTCGGAGCTAATGAAGATGACGGAGT 1442
TcAm-2_KF247315             GGAGATGGATATGCCGATATTTCCCTCGGAGCTAATGAAGATGATGGAGT 1442
NM_001114376.1Tcastaneum    GGAGATGGATATGCCGATCTTTCCCTCGGAGCTAATGAAGATGACGGAGT 1450
                            ******************.************************* *****

TcAm-1_KF247314             GATTGCCATTCATGTTAATGCCAAATTG---------------------- 1470
TcAm-3_KF247316             GATTGCCATTCATGTTAATGCCAAATTG---------------------- 1470
TcAm-2_KF247315             GATTGCTATTCATGTTAATGCCAAATTG---------------------- 1470
NM_001114376.1Tcastaneum    GATTGCCATTCATGTTAATGCCAAATTGTAAACAAATAAACTGTATTTAT 1500
                            ****** *********************

TcAm-1_KF247314             ------------------
TcAm-3_KF247316             ------------------
TcAm-2_KF247315             ------------------
NM_001114376.1Tcastaneum    TACAAAAAAAAAAAAAAA 1518
```

Figure 28 (Cont.)



```
NM_001114376.1         MHFKPILVLCLATLALGQKDPHFAADRNSIVHLFEWKWSDIADECERFLAPKGFGGVQIS 60
TcAm-2_KF247315        MHFKPILVLCLATLALGQKDPHFAADRNSIVHLFEWKWSDIADECERFLAPKGFGGVQVS 60
TcAm-3_KF247316        MHFKPILVLCLATLALGQRDPHFAADRNSIVHLFEWKWSDIADECERFLAPKGFGGVQIS 60
TcAm-1_KF247314        MHFKPILVLCLATLALGQKDPHFAADRNSIVHLFEWKWSDIADECERFLAPKGFGGVQIS 60
                       ******************:***************************************:*

NM_001114376.1         PPNENLVVTSSNRPWWERYQPVSYILNTRSGDEAALADMISRCNAVGVRIYVDTVINHMT 120
TcAm-2_KF247315        PPNENLVVTSSNRPWWERYQPVSYILNTRSGDEAALADMISRCNAVGVRIYVDTVINHMT 120
TcAm-3_KF247316        PPNENLVVTSSNRPWWERYQPVSYILNTRSGDETALADMISRCNAVGVRIYVDTVINHMT 120
TcAm-1_KF247314        PPNENLVVTSSNRPWWERYQPVSYILNTRSGDETALADMISRCNAVGVRIYVDTVINHMT 120
                       *********************************:**************************

NM_001114376.1         GMGGTGTAGSQADRDGKNYPAVPYGSGDFHDSCTVNNYQDASNVRNCELVGLADLNQGSD 180
TcAm-2_KF247315        GMGGTGTAGSQADRDGKNYPAVPYGSGDFHDSCTVNNYQDASNVRNCELVGLADLNQGSD 180
TcAm-3_KF247316        GMGGTGTAGSQADRDGKNYPAVPYGSGDFHDSCTVNNYQDASNVRNCELVGLADLNQGSD 180
TcAm-1_KF247314        GMGGTGTAGSQADRDGKNYPAVPYGSGDFHDSCTVNNYQDASNVRNCELVGLADLNQGSD 180
                       ************************************************************

NM_001114376.1         YVRSKIIEYMNHLVDLGVAGFRVDAAKHMWPADLEAIYASLKNLNTDHGFLDGQKPFIFQ 240
TcAm-2_KF247315        YVRSKIIEYMNHLVDLGVAGFRVDAAKHMWPADLEAIYGSLKNLNTDHGFLDGQKPFIFQ 240
TcAm-3_KF247316        YVRSKIIEYMNHLVDLGVAGFRVDAAKHMWPADLEAIYGSLKNLNTDHGFLDGQKPFIFQ 240
TcAm-1_KF247314        YVRSKIIEYMNHLVDLGVAGFRVDAAKHMWPADLEAINGSLKNLNTDHGFLDGQKPFIFQ 240
                       ************************************* .*********************

NM_001114376.1         EVIDLGGEAISKHEYTGFGTVIEFQYGLSLGNAFQGGNQLANLANWGPEWNLLDGLDAVA 300
TcAm-2_KF247315        EVIDLGGEAISKHEYTGFGTVIEFQYGLSLGNAFQGGNQLANLANWGPEWNLLDGLDAVA 300
TcAm-3_KF247316        EVIDLGGEAISKHEYTGFGTVIEFQYGLSLGNAFQGGNQLANLANWGPEWNLLDGLDAVA 300
TcAm-1_KF247314        EVIDLGGEAISKHEYTGFGTVIEFQYGLSLGNAFQGGNQLANLANWGPEWNLLDGLDAVA 300
                       ************************************************************

NM_001114376.1         FIDNHDNQRTGGSQILTYKNPKPYKMAIAFMLAHPYGTTRLMSSFAFDNNDQGPPQDGAG 360
TcAm-2_KF247315        FIDNHDNQRTGGSQILTYKNPKPYKMAIAFMLAHPYGTTRLMSSFAFDSNDQGPPQDGAG 360
TcAm-3_KF247316        FIDNHDNQRTGGSQILTYKNPKPYKMAIAFMLAHPYGTTRLMSSFAFDSNDQGPPQDGAG 360
TcAm-1_KF247314        FIDNHDNQRTGGSQILTYKNPKPYKMAIAFMLAHPYGTTRLMSSFAFDSNDQGPPQDGAG 360
                       ************************************************.***********

NM_001114376.1         NLISPSINDDGTCGNGYVCEHRWRQIFNMVGFRNAVQGTGIENWWSDGNQQIAFGRGNKG 420
TcAm-2_KF247315        NLISPSINDDGTCGNGYVCEHRWRQIFNMVGFRNAVQGTGIENWWSDGNQQIAFGGGNKG 420
TcAm-3_KF247316        NLISPSINDDGTCGNGYVCEHRWRQIFNMVGFRNAVQETGTENWWSDGNQQIAFGRGNKG 420
TcAm-1_KF247314        NLISPSINDDGTCGNGYVCEHRWRQIFNMVGFRNAVQETGTENWWSDGNQQIAFGRGNKG 420
                       ************************************* ** ************** ****

NM_001114376.1         FVAFTIGYDLNQHLQTGLPAGSYCDVISGNAENGSCSGKTITVGGDGYADLSLGANEDDG 480
TcAm-2_KF247315        FVAFTIGYDLNQHLQTGLPAGSYCDVISGNAENGSCSGKTITVGGDGYADISLGANEDDG 480
TcAm-3_KF247316        FVAFTIGYDLNQHLQTGLPAGSYCDVISGNAENGSCSGKTITVGGDGYADISLGANEDDG 480
TcAm-1_KF247314        FVAFTIGYDLNQHLQTGLPAGSYCDVISGNAENGSCSGKTITVGGDGYADISLGANEDDG 480
                       **************************************************:*********

NM_001114376.1         VIAIHVNAKL 490
TcAm-2_KF247315        VIAIHVNAKL 490
TcAm-3_KF247316        VIAIHVNAKL 490
TcAm-1_KF247314        VIAIHVNAKL 490
                       **********
```

Figure 29 Comparison of deduced amino acid sequences of three cDNAs (Gene Bank Accession NumberKF247314, KF247315, KF247316) cloned from *Tribolium castaneum,* encoding three putative α-amylases, and the NCBI database (NCBI Reference Sequence: NM_001114376.1).The first seventeen amino acids (highlighted in dots line) represent the signal peptide. Active site residues (Asp204, Glu241, and Asp303) are shown in white boxes. The four amino acids are calcium (Asn117, Arg165, Asp174, and His218) binding site (highlighted in gray box and underline). The three amino acids are chloride binding site (Arg202, Asn304, and Arg340) (highlighted in grey box)

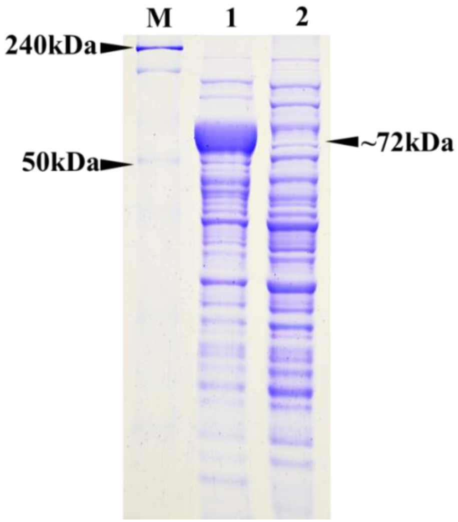


Figure 30 10% SDS-PAGE gel of pET32a (+) – 1,470 bp α-amylase recombinant cells was induced with 1mM IPTG at 37 °C for 1hr. Lane M: protein ladder, lane 1: 1470 bp α-amylase - pET32a (+) recombinant cells, resulted in the intense band of approximately 72 kDa (TcAm: 53.3 kDa + Thioredoxin/Hitidine tag from vector: 18.7 kDa)

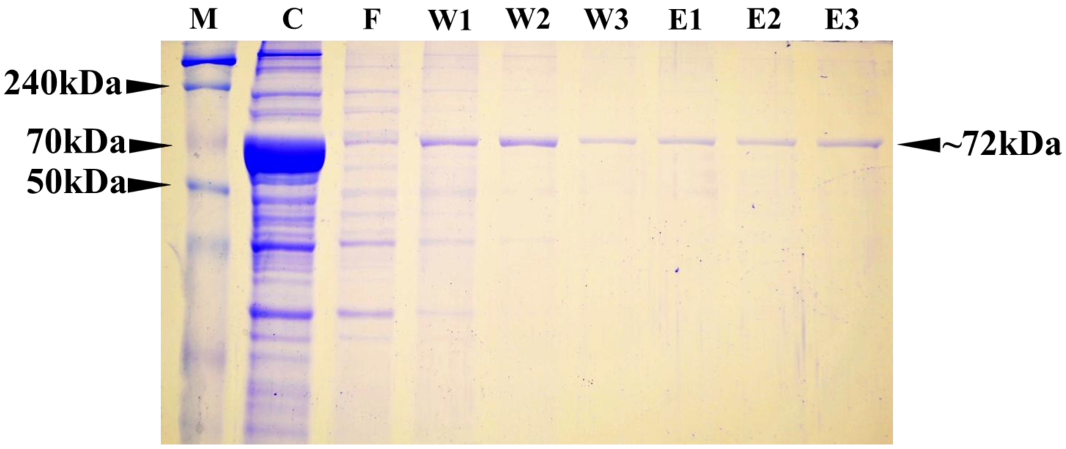
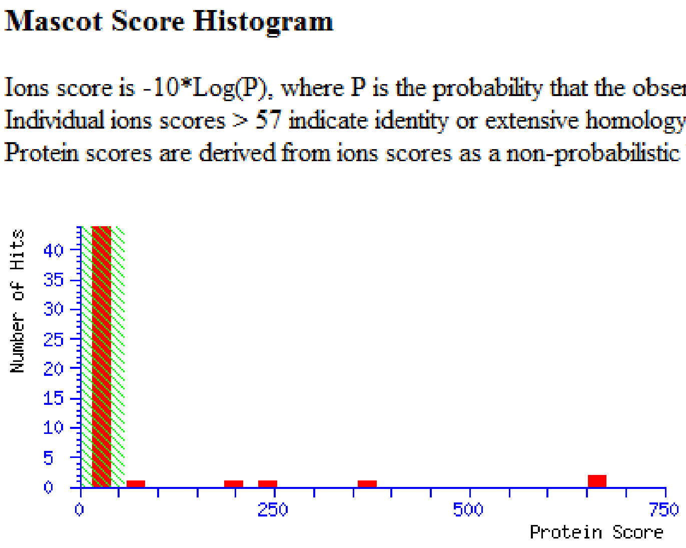


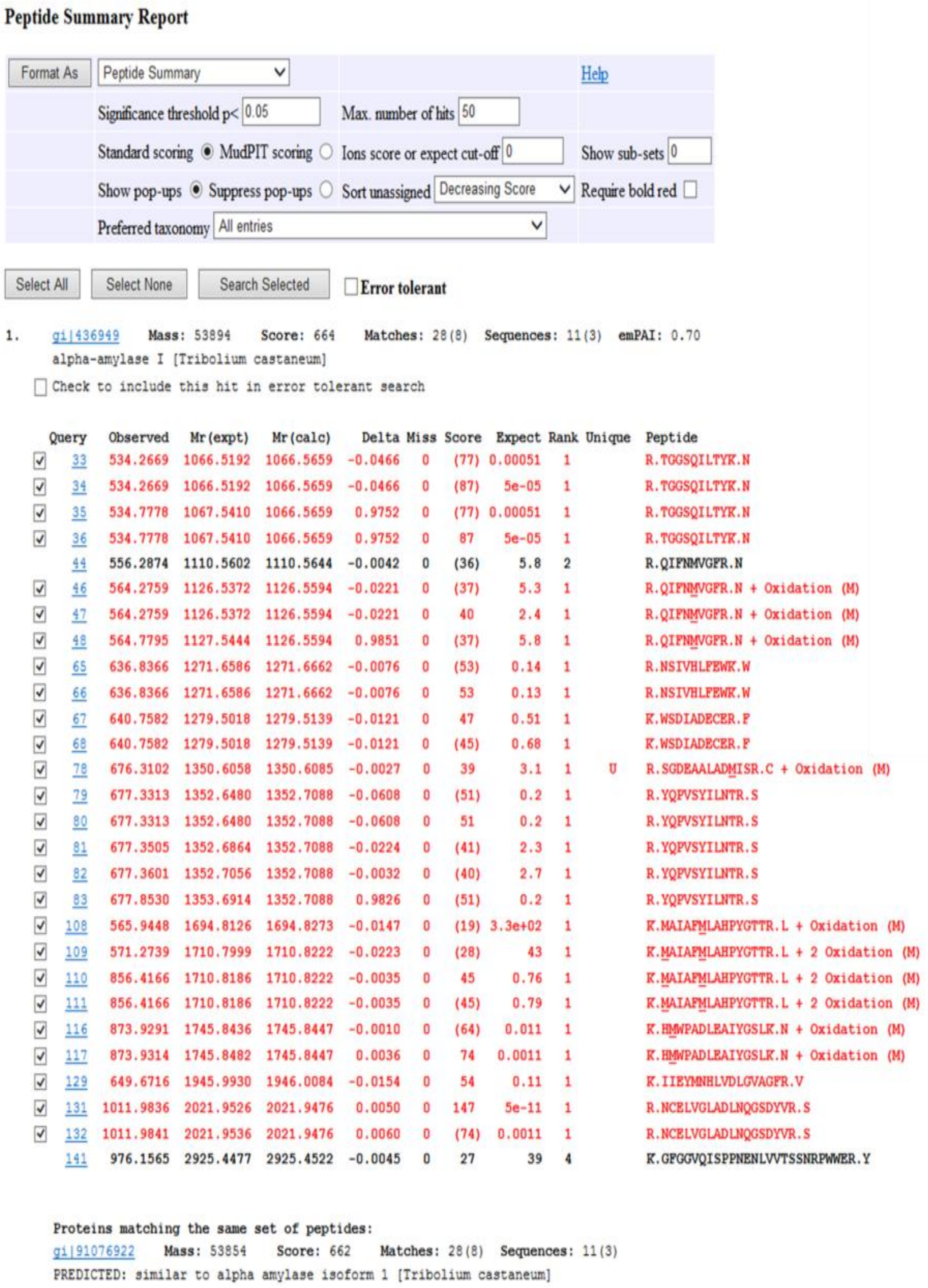
Figure 31 10% SDS-PAGE of purified TcAm using Ni^{2+} affinity chromatography. Lane M: prestainned protein ladder, Lane C: crude extract TcAm, Lane F: Flow through fraction, Lane W1-W3: washed fraction, Lane E1-E3: eluted fraction



Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 57 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Figure 32 Mascot search of LC-MS/MS result showing that the tryptic digests of 53.3 kDa protein have exactly matched to α-amylase of *Triboliumcastaneum*



Peptide Summary Report

Format As: Peptide Summary

Significance threshold p< 0.05

Max. number of hits 50

Standard scoring ● MudPIT scoring ○

Ions score or expect cut-off 0

Show sub-sets 0

Show pop-ups ● Suppress pop-ups ○

Sort unassigned: Decreasing Score

Require bold red ☐

Preferred taxonomy: All entries

☐ Error tolerant

1. gi|436949 **Mass:** 53894 **Score:** 664 **Matches:** 28(8) **Sequences:** 11(3) **emPAI:** 0.70
alpha-amylase I [Tribolium castaneum]

☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑	33	534.2669	1066.5192	1066.5659	-0.0466	0	(77)	0.00051	1		R.TGGSQILTYK.N
☑	34	534.2669	1066.5192	1066.5659	-0.0466	0	(87)	5e-05	1		R.TGGSQILTYK.N
☑	35	534.7778	1067.5410	1066.5659	0.9752	0	(77)	0.00051	1		R.TGGSQILTYK.N
☑	36	534.7778	1067.5410	1066.5659	0.9752	0	87	5e-05	1		R.TGGSQILTYK.N
	44	556.2874	1110.5602	1110.5644	-0.0042	0	(36)	5.8	2		R.QIFNMVGFR.N
☑	46	564.2759	1126.5372	1126.5594	-0.0221	0	(37)	5.3	1		R.QIFNMVGFR.N + Oxidation (M)
☑	47	564.2759	1126.5372	1126.5594	-0.0221	0	40	2.4	1		R.QIFNMVGFR.N + Oxidation (M)
☑	48	564.7795	1127.5444	1126.5594	0.9851	0	(37)	5.8	1		R.QIFNMVGFR.N + Oxidation (M)
☑	65	636.8366	1271.6586	1271.6662	-0.0076	0	(53)	0.14	1		R.NSIVHLFEWK.W
☑	66	636.8366	1271.6586	1271.6662	-0.0076	0	53	0.13	1		R.NSIVHLFEWK.W
☑	67	640.7582	1279.5018	1279.5139	-0.0121	0	47	0.51	1		K.WSDIADECER.F
☑	68	640.7582	1279.5018	1279.5139	-0.0121	0	(45)	0.68	1		K.WSDIADECER.F
☑	78	676.3102	1350.6058	1350.6085	-0.0027	0	39	3.1	1	U	R.SGDEAALADMISR.C + Oxidation (M)
☑	79	677.3313	1352.6480	1352.7088	-0.0608	0	(51)	0.2	1		R.YQPVSYILNTR.S
☑	80	677.3313	1352.6480	1352.7088	-0.0608	0	51	0.2	1		R.YQPVSYILNTR.S
☑	81	677.3505	1352.6864	1352.7088	-0.0224	0	(41)	2.3	1		R.YQPVSYILNTR.S
☑	82	677.3601	1352.7056	1352.7088	-0.0032	0	(40)	2.7	1		R.YQPVSYILNTR.S
☑	83	677.8530	1353.6914	1352.7088	0.9826	0	(51)	0.2	1		R.YQPVSYILNTR.S
☑	108	565.9448	1694.8126	1694.8273	-0.0147	0	(19)	3.3e+02	1		K.MAIAFMLAHPYGTTR.L + Oxidation (M)
☑	109	571.2739	1710.7999	1710.8222	-0.0223	0	(28)	43	1		K.MAIAFMLAHPYGTTR.L + 2 Oxidation (M)
☑	110	856.4166	1710.8186	1710.8222	-0.0035	0	45	0.76	1		K.MAIAFMLAHPYGTTR.L + 2 Oxidation (M)
☑	111	856.4166	1710.8186	1710.8222	-0.0035	0	(45)	0.79	1		K.MAIAFMLAHPYGTTR.L + 2 Oxidation (M)
☑	116	873.9291	1745.8436	1745.8447	-0.0010	0	(64)	0.011	1		K.HMWPADLEAIYGSLK.N + Oxidation (M)
☑	117	873.9314	1745.8482	1745.8447	0.0036	0	74	0.0011	1		K.HMWPADLEAIYGSLK.N + Oxidation (M)
☑	129	649.6716	1945.9930	1946.0084	-0.0154	0	54	0.11	1		K.IIEYMNHLVDLGVAGFR.V
☑	131	1011.9836	2021.9526	2021.9476	0.0050	0	147	5e-11	1		R.NCELVGLADLNQGSDYVR.S
☑	132	1011.9841	2021.9536	2021.9476	0.0060	0	(74)	0.0011	1		R.NCELVGLADLNQGSDYVR.S
	141	976.1565	2925.4477	2925.4522	-0.0045	0	27	39	4		K.GFGGVQISPPNENLVVTSSNRPWWER.Y

Proteins matching the same set of peptides:
gi|91076922 **Mass:** 53854 **Score:** 662 **Matches:** 28(8) **Sequences:** 11(3)
PREDICTED: similar to alpha amylase isoform 1 [Tribolium castaneum]

Figure 33 **Identification of peptide mass fingerprints of α-amylase protein that was induced with 1 mM IPTG at 37 °C for 2 hr by LC-MS. The protein has exactly matched to α-amylase of *Tribolium castaneum***

Enzymatic activity and biochemical properties of recombinant *Tribolium castaneum* α-amylase

In our research, the pET32a(+) expression vector was employed to produce recombinant TcAm in over expression protein condition. The pET32a(+) expression vector is designed for cloning and high-level expression of peptide sequences fused with the 109aa thioredoxin (Trx-tag) protein. Numerous studies showed that these fusion tags enhance solubility and folding in two paths: the tags provide the fusion to a polypeptide creating higher soluble polypeptide, and provide the fusion to an enzyme catalyzing disulfide bond formation. Seonghun Kim and Sun Bok Lee,(2008) reported that the highest solubility of recombinant proteins was observed in (Trx-tag) fusion protein in *E. coli* BL21(DE3) pLysS, the host cell. Correlated with our result, the recombinant TcAm was completely dissolved in PBS buffer.

The amylolytic activity was determined by running the purified recombinant protein on the native polyacrylamide gel (10%) soaking with 1% (w/v) soluble starch. The activity was subsequently visualized with iodine solution. The clear zone ondarkbackground was obtained on native gel indicating the location of the purified enzyme, which possesses the amylolytic activity. This result was also concordant with the location of the purified enzyme on native gel stained with Coomassie brilliant blue.

The zymogram analysis indicated the existence of a single band of TcAm α-amylases from both the crude extract and the purified enzyme (Figure 34).Approximately 50-70 kDa ofthe purified α-amylases were obtained from the result of zymogram. However, these values were different from the result obtained from SDS-PAGE, which showed that the size of TcAm was at 72 kDa. This could be because ofthe native conditions, the charge of each protein, which depended on the isoelectric point and the pH that mainly influenced the mobility during electrophoresis (Claudia Arndt, et al., 2012).

The purified TcAm showed 6818.2 U/mg specific activities (Table 2) to the 2-Chloro-4-nitrophenyl-α-D-maltotrioside as a substrate at 50 °C, 30 min. The recombinant TcAm enzyme showed that the optimal temperature for the activitywas at 50 °C (Figure 36). Similar temperature optima from 50-55 °C have been reported for native purified α-amylase of *Tribolium castaneum* (Sineenat Kembubpha, 2011). Nevertheless, the different temperature optima from 40–45 °C have been reported in other insect native α-amylases as well (E. Mendiola-Olaya, et al., 2000: H. Podoler, et al., 1971: B. Dojnov, et al., 2008).

From 20–80 °C, the activity was retained up to more than 80% before starting to decrease at higher temperature. The thermal stability of TcAm was also determined as shown in Figure 37. The results showed that the thermal stability decrease occurred when the protein was at 60-70 °C for 15 min. However, the protein was stable when heated to 30, 40 and 50 °C as it showed the relative activity up to more than 80% for 2 hr.

Furthermore, the effect of pH on the purified enzyme was determined as shown in Figure 38. The activity was obtained in the range of buffer from pH 6.6 – 9 and the optimal pH was at pH 7. Various metal ions and reagents were tested as inhibitors or activators at optimal conditions (Figure 39). The results showed that the enzyme activity was significantly enhanced with 10 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ and Imidazole under optimal condition. This was probably caused by the fact that most α-amylases are Ca^{2+} dependent enzyme. Moreover, $MgCl_2$ and $MnCl_2$ are more potent activators compared to $CaCl_2$ ions in native purified α-amylase of *Rhyzopertha dominica* (Smriti Priya, 2010). In addition, imidazole has been discovered to be an effective enzyme activity inducer as well, since 10 mM Imidazole has previously reported to induce the activity of tyrosinase (Ellis L. Kline, 2005).On the other hand, no effect observed on the TcAm activity in the reaction with the presence of NaCl, EDTA, and KCl. This could be possibly explained by the result that the recombinant TcAm showed activity in the partial removal of Ca^{2+} condition. Normally, the monovalent ion could destabilize the enzyme by causing the partial removal of Ca^{2+} from the protein or its displacement, since calcium ions are important for the catalytic activity (G.E. Smolka, et al., 1971). Indicated that, the catalytic activity of recombinant TcAm was found by removal of Ca^{2+} condition and the monovalention has no effect on recombinant TcAm.

Actually, the native purified α-amylase activity from *Tribolium castaneum* has been studied and reported (Sineenat Kembubpha, 2011). However, the results were less specific than ours because the 3, 5-dinitrosalicylate (DNS), α-amylase substrate, employed in their study was less sensitive. According to our research, 2-Chloro-4-nitrophenyl-α-D-maltotrioside, the α-amylase substrate which possesses higher sensitivity than DNS was selected to determine the enzyme activity. In contrast, the results showed that the highest activity of enzyme was achieved at 50 °C, pH 7, different from the report of Sineenat Kembubpha, 2011, which indicated the optimal condition at 55 °C, pH 6 and 55 °C, pH 5.5 of native purified Tribolium castaneum α-amylase from two sources.Nevertheless, the thermal stability of the purified α-amylase from *Tribolium castaneum* was correlated with recombinant TcAm and concordant withnumerous reported results (Baker, 1983; Baker, 1987).

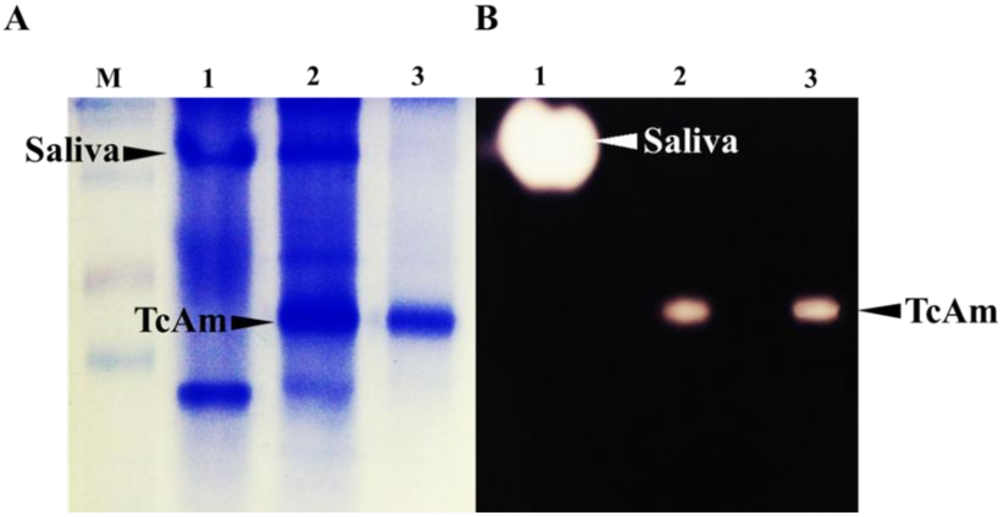


Figure 34 Starch zymogram analysis of recombinant TcAm under non-denature condition. Native PAGE stained with Coomassie brilliant blue R-250 (A) M: pre-stained protein ladder, Lane1: Human saliva, Lane2: crude extract TcAm, Lane3: purified TcAm. Enzyme activity was determined by starch zymogramstaining with 3% KI-1.3% I2 solution (B) Lane1: Human saliva, Lane2: crude extract TcAm, Lane3: purified TcAm

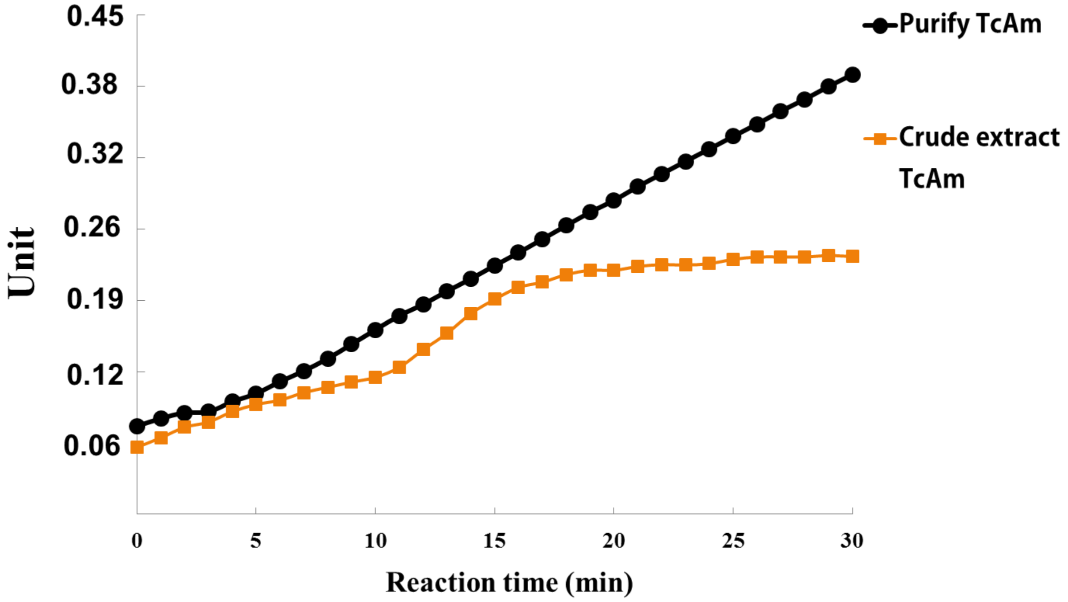


Figure 35 **Kinetic of recombinant TcAm measured with2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate at 50 °C, 30 min runtime and 1 min interval**

Table 1 **Activity of recombinant *Tribolium castaneum* α-amylase was determined with the 2-Chloro-4-nitrophenyl-α-D-maltotrioside substrate at 50 °C 30 min**.

	Total Activity (U)	**Total Protein (mg)**	**Specific activity (U/mg)**	**Purification fold**	**Yield (%)**	**Purity (%)**
Crude extract	**366.95**	**0.897**	**409.08**	**1**	**100**	**53.69**
Purified TcAm	**332.95**	**0.437**	**761.9**	**1.8**	**90.73**	**100**

Units: µmoles min^{-1}

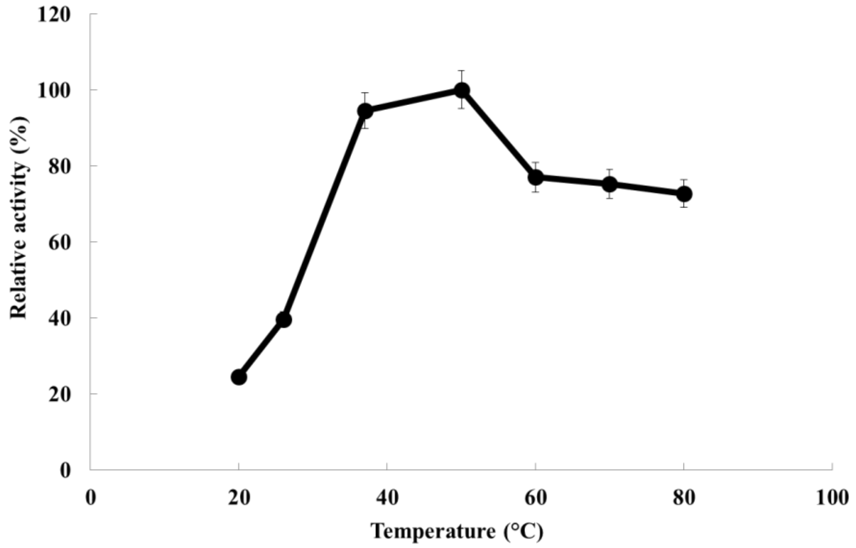
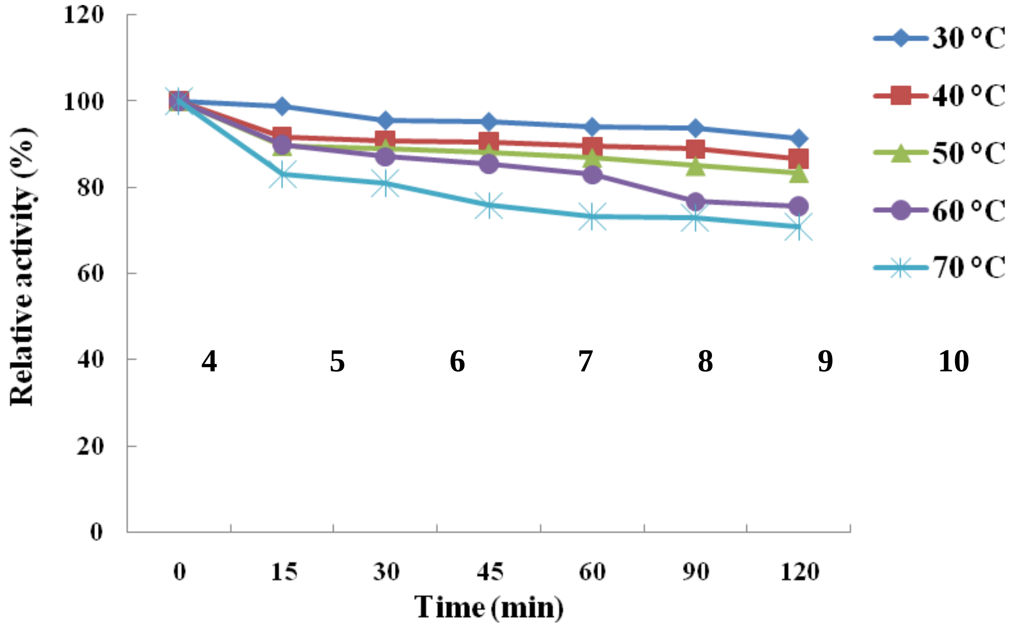


Figure 36 Relative enzyme activity (% of maximum) of purified TcAm measured at different temperatures ranging from 20-80 °C for 30 min



4 5 6 7 8 9 10 11

Figure 37 Determination of the thermal stability at different temperatures for different time points. Thermal stability of the recombinant TcAm was determined by measuring activity under optimum conditions, after incubation at various temperatures (30, 40, 50, 60 and 70 °C) for different periods of time ranging from 0 –120 min

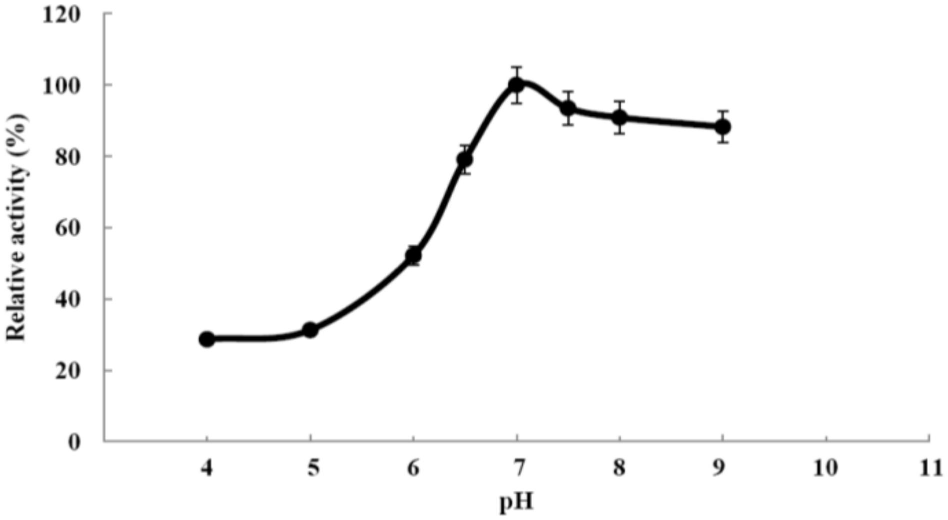


Figure 38 Effect of pH on enzyme activity was assayed at different pH values range from 4.0-9.0 using 2-Chloro-4-nitrophenyl-α-D-maltotrioside as a substrate

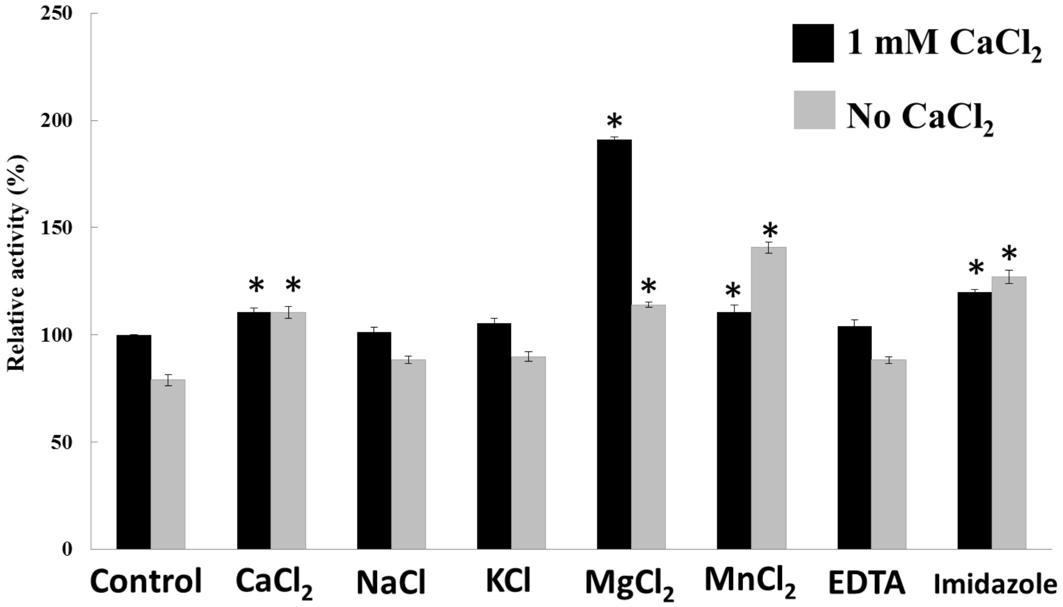


Figure 39 Effects of some metal ions and reagent on the TcAm activity was determined using 2-Chloro-4-nitrophenyl-α-D-maltotrioside as a substrate at 50 °C, pH 7 for 30 min. Black bars: 10 mM of each ions, Gray bars: 1 mM $CaCl_2$ + 10 mM of each ions