CHAPTER IV

RESULTS

4.1 Expression level of alpha glucosidase *(AG)* in *Apis florea*

Honeybees from 3 different stages (egg, nurse bee, and forager bee) were sampled. Total RNA was isolated. The quality of total RNA was determined by (1) native agarose gel and (2) formaldehyde gel. The 18S and 28S rRNA bands were detected on 1.2% agarose gel (Fig. 4.1 A) while the 28S RNA band was visible on formaldehyde gel (Fig. 4.1 B).
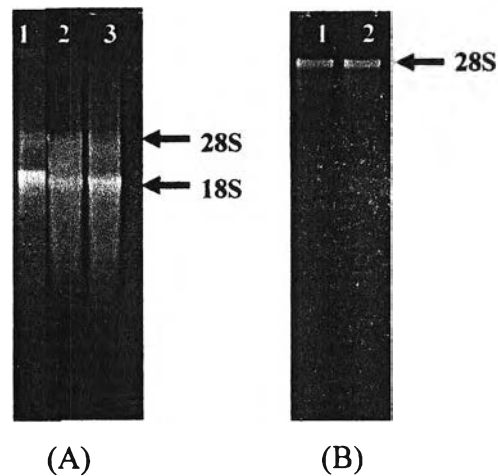

Figure 4.1 Total RNA extracted from heads of *A. florea* at different stages on native agarose gel (A) and formaldehyde gel (B).

Lane 1 (A):	total RNA of egg
Lane 2 (A):	total RNA of nurse bee
Lane 3 (A):	total RNA of forager bee
Lanes 1 – 2 (B):	total RNA of forager bee

In order to determine the expression level of *AG* by RT – PCR, 200 ng of RNA sample (egg, nurse bee, and forager bee) were used for 1 reaction. Primers were designed from the *AG* cDNA sequence of *A. mellifera* as described in Materials and Methods. The primers for determination of expression are FW1/ R1 primers. Under

the optimum condition of RT – PCR, the expression profile of *AG* was obtained (Fig. 4.2). The quantity of products was assayed due to intensity of the bands by Quanlity one software (Table 4.1). The result presented that the expression level of *AG* in three stages (egg, nurse bee, forager bee) was different. There was no amplified product from egg RNA (Fig. 4.2, lanes 1 – 2) and small amounts of amplified product from nurse bee RNA (Fig. 4.2, lanes 3 – 4). The highest amount of amplified products was obtained from forager bees (Fig. 4.2, lanes 5 – 6).
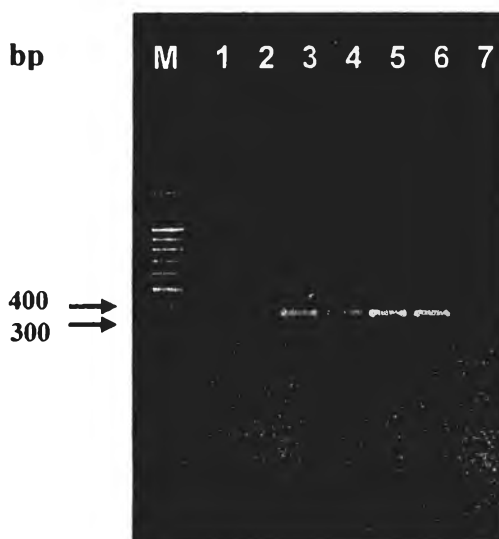

Figure 4.2 Expression profile of *AG*.

Lane M:	100 bp ladder marker
Lanes 1 - 2:	amplified products from egg RNA
Lanes 3 - 4:	amplified products from nurse bee RNA
Lanes 5 - 6:	amplified products from forager bee RNA
Lane 7:	negative control

Table 4.1 Intensity of amplified product bands from Fig. 4.2.

Stage	Average volume intensity*mm^2
Eggs	16.082
Nurse bees	386.633
Forager bees	760.589

As control experiments, primers specific to elongation factor gene (*EF*) in *A. cerana* and *28S RNA* in *A. mellifera* were designed. Under the optimum condition, the products of 200 bp (*EF*) and 350 bp (*28S RNA*) were obtained from all samples, respectively (Fig. 4.3).
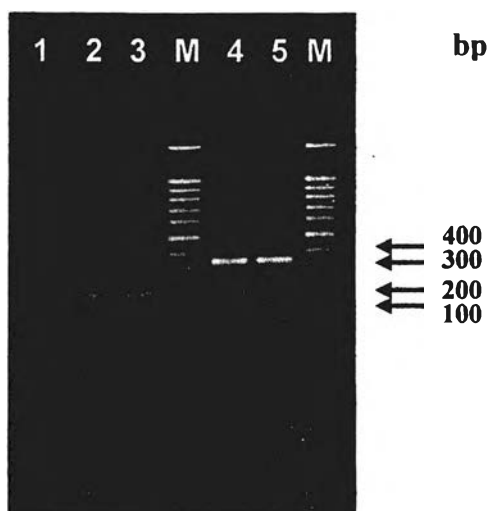

Figure 4.3 Control experiment by using primers from *EF* and *28S RNA* genes. Total RNA for all reactions were from forager bee.

Lane 1:   negative control

Lanes 2-3: RT – PCR product by using *EF* primers

Lanes 4-5: RT – PCR product by using *28S RNA* primers

Lane M: 100 bp ladder marker

4.2 The cDNA sequence

Total RNA of forager bee was amplified by 3 pairs of primers for RT – PCR. The sizes of RT – PCR product were 350 bp from FW1/ R1 primers (Fig. 4.4 A), 1,000 bp from FW1/ R2 primers (Fig. 4.4 B), and 850 bp from FW2/ R3 primers (Fig. 4.4 C). Three bands (200, 300, and about 380 bp; Fig. 4.4 D) were obtained from FW3/ R3 primers but only the 200 bp product was excised from the agarose gel, purified, and sequenced (Fig. 4.4 E).

(A)   (B)   (C)

(D)   (E)
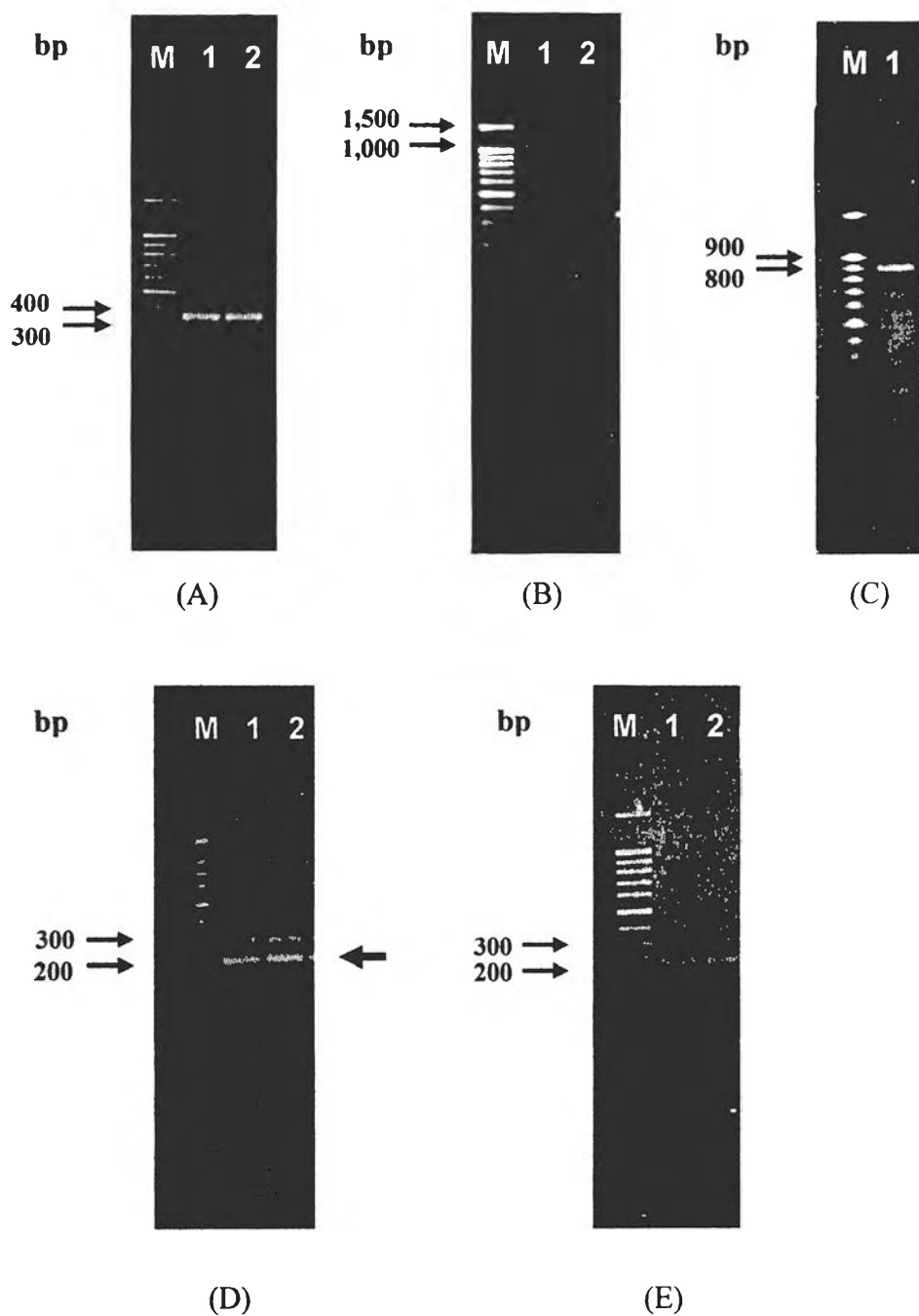
Figure 4.4 RT - PCR product amplified by 3 different pairs of primers.

Lane M (all Figs.):	100 bp ladder marker
Lanes 1 – 2 (A):	the 350 bp product by FW1/ R1 primers
Lanes 1 – 2 (B):	the 1,000 bp product by FW1/ R2 primers
Lane 1 (C):	the 850 bp product by FW2/ R3 primers
Lanes 1 – 2 (D):	the 200, 300, and about 380 bp products by FW3/ R3 primers
Lanes 1 – 2 (E):	the excised and purified target band (200 bp)

The nucleotide sequences and deduced amino acid sequences of *AG* were aligned (Figs. 4.5 and 4.6) by using Clustal W.

```
AG.Af.nuc      T1CGACTTCTAGTTGGTAGCATGAAGGCGGTAATCGTGTTTTGCCTT-ATGGCATTGTCC 59
AG.Am.nuc      TTCGACTTCTAGTTGGTAGCATGAAGGCAGTAATCGTATTTTGCCTT-ATGGCATTGTCC 59
AG.Dm.nuc      TTGTAGTGAAAATAGCTTTCATTTTGAGTGTGGGCCTAGTAGGCAT--ATTG----GCCC 54
maltase.Cs.nuc TATAAAAGAAAAATGATTCCATTTAAAAAATTAACAATTTTACTATCAATTGCAT-GTTC 59
maltase.Am.nuc -------------------ATGAAGAGCCTCGTCGTGGTCGTACTT------CTGCTC 33
ScrA.Ls.nuc    GTTTAGTTTGGGCTTATCAAACGTTAGGTGCAGTCGGTATGGGGATTTTTGGCCTGGGTT 60
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AG.Af.nuc      ATT-GTGGACGCAGCATGGAAGCCACTCCCTGAAA--ACTTGAAGGAGGACTTGATCTTG 116
AG.Am.nuc      ATT-GTGGACGCAGCATGGAAGCCGCTCCCTGAAA--ACTTGAAGGAGGACTTGATCGTG 116
AG.Dm.nuc      ATA-AGCACCAGTCAAAGGAGCTGGATGCGAAATATAATTGGTGGCAGCACGAGGTCTTC 113
maltase.Cs.nuc TGT-ATTGGCAGCACCTGAAGGTGCACGTGAAAAA-GATTGGTGGGAAATTGGAAACTTT 117
maltase.Am.nuc GCG-GTCGGCCTTG-GCGCCGGCCAAAACAACAAG-GGTTGGTGGAAGAACGCGATCTTC 90
ScrA.Ls.nuc    ATTCAGCAATTGTCTTAACTGGCTTACATCAAAGCTTCCCGGCAATTGAAACGACACTTT 120
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AG.Af.nuc      TATCAGGTTTACCCGA----GGAGCTTCAAGGATAGCAATGGAGATGGTATTGGTGATAT 172
AG.Am.nuc      TATCAGGTCTACCCGA----GAAGCTTCAAGGATAGCAATGGAGATGGTATTGGTGATAT 172
AG.Dm.nuc      TATCAGATCTATCCGA----GATCCTTTCAGGACAGCAATGGTGATGGTATTGGTGATCT 169
maltase.Cs.nuc TATCAAGTCTATCCAC----GAAGTTTCATGGATTCTGATGGCGATGGTGTTGGCGATTT 173
maltase.Am.nuc TATCAGGTATATCCCC----GCAGTTTCATGGATTCCAATAGTGATGGCATCGGGGATTT 146
ScrA.Ls.nuc    TGGCAGATATTGCCAAAACTGGTGGATCGTTTATTTTTCCCGTTGCAGCGATGGCAAATA 180
                *  **  *  *  **       *       *       *       *     **  *
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```
AG.Af.nuc      CATAGGTATTAAAGAAAAATTGGATCAT-TTTCTCGAAATGGGCGTCGACATGTTTTGGT 231
AG.Am.nuc      CGAAGGTATTAAAGAAAAATTGGATCAT-TTTCTCGAAATGGGGGTCGACATGTTTTGGT 231
AG.Dm.nuc      TCAAGGTATTACTTCTAGGCTACAGTAC-TTCAAGGATACGGGCATCACGTCCGTATGGT 228
maltase.Cs.nuc GAAAGGAATTTCAGAAAAAGTCGGTTAT-TTAAAGGAAATCGGCATGGATGGTGTTTGGC 232
maltase.Am.nuc AAAAGGTATTAAGGATAAGCTTTCACAC-TTCATCGAATCTGGAATAACAGCGATATGGT 205
ScrA.Ls.nuc    TTGCTCAAGGGGCTGCAACTTTCGCTGTATTCTTCGTTACTAAGAATAAACAACAAAAGT 240
                        *       *    *        **   *                    *
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```
AG.Af.nuc      TATCCCCTATTTATCCAAGCCCTATGGTCGATTTTGGTTACGACATTTCCAATTACACCG 291
AG.Am.nuc      TATCCCCTATTTATCCAAGCCCTATGGTCGATTTTGGTTACGACATTTCGAATTACACCG 291
AG.Dm.nuc      TGAGTCCCATTTATGAGTCACCAATGGTAGACTTTGGATACGATATATCTAACTATACAA 288
maltase.Cs.nuc TTTCACCGATTTTTGATTCACCTATGGCAGATTTTGGTTATGACATTTCAAATTTCACCA 292
maltase.Am.nuc TATCACCAATTAATCGAAGTCCTATGGTAGATTTTGGATACGATATATCTGACTTTAAAG 265
ScrA.Ls.nuc    CATTAACGACTTCTGCTGGGATTTCTGC-GATGTTGGGAATTACTGAACCAGCATTATTT 299
                   *   *  *          *   **  ****   *   *       *      *
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```
AG.Af.nuc      ACGTTCATCCCATATTTGGCACCATATCAGATTTAGATGACCTAGTCAGTGCTGCACATG 351
AG.Am.nuc      ACGTTCATCCCATATTTGGCACCATATCAGACTTAGATAATCTAGTCAGTGCTGCACATG 351
AG.Dm.nuc      ATATACAGCCGGAATATGGCACCCTTGAGGACTTTGACGCCTTGATAGCCAAGGCCAATG 348
maltase.Cs.nuc AAGTCTTCCCTCAATTCGGAGACTTGTCTTCAATTGATGAACTTGTAGCGGAATTCAATA 352
maltase.Am.nuc ATGTAGATCCAATATTTGGTACTATAAAAGATCTTGAAGATCTCACTGCAGAAGCGAAGA 325
ScrA.Ls.nuc    GGGG_.TATTTAAAATTGAAGTTTC--CATTCTTTATTGGTTTAATTGCATCAGGAATCT 357
                   *  *             *        *       *
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```
AG.Af.nuc      AGAAAGGACTGA--AGATAATCTTGGATTTCGTTCCGAATCATACATCTGATCAACACAA 409
AG.Am.nuc      AGAAAGGATTGA--AGATAATCTTGGATTTCGTCCCGAATCATACATCTGATCAACACGA 409
AG.Dm.nuc      AACTGGGCGTGA--AAGTTATTTTGGACTTTGTTCCCAATCACAGCTCAAATAAGCATCC 406
maltase.Cs.nuc AAAAAGATATGA--AACTCATTCTGGACTTTGTTCCAAATCATACAAGTGACCAATGTGA 410
maltase.Am.nuc AACAGAATTTAA--AGGTTATTCTAGATCTTGTCCCTAATCATACTTCTGATCAACATAA 383
ScrA.Ls.nuc    CATCGTTTATTATTGGTTTATTACATGTTTTATCAGTATCAATGGGACCTGCAGGAATTA 417
                 *  *     *  r*    *   *     *   *
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AG.Af.nuc      ATGG-TTCCAGTTGAGTTTGAAAAACGTTGAACCT---------------TATAACAA 451
AG.Am.nuc      ATGG-TTCCAGTTGAGTTTGAAAAACATTGAACCT---------------TATAACAA 451
AG.Dm.nuc      CTGG-TTC--ATAAAGTCAGTAGCCCGA-GAGCCAGG--------------GTACGAGGA 448
maltase.Cs.nuc GTGG-TTC--AAAAAATCAATTCAGCGT-GATCCTGA-------------GTACAATGA 452
maltase.Am.nuc ATGG-TTCCAAATGAGTATAAATAATACTAATAATAATAATAC--TAATAAATATAAAGA 440
ScrA.Ls.nuc    TTGGGTTTATTGCGATTGCACCTAAGAGCATCCCTAGTTTTATGATGGGAGCTATTATTA 477
                *** **       *  *                              **  *   *
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Figure 4.5 (continued)

```
AG.Af.nuc      CTATTATATTTGGC---ATCCAGGAAAAAT------TGTAAAT---GGTAAACGTGTTCC 499
AG.Am.nuc      CTATTACATTTGGC---ATCCAGGAAAAAT------TGTAAAT---GGCAAACGTGTTCC 499
AG.Dm.nuc      TTTCTATGTGTGGG---AGGATGGTATTCT---CCTGGAGAAC---GGAACTCGTGTGCC 499
maltase.Cs.nuc TTACTATATTTGGC---ATCCGGGTAAGCCAAATCCTGATGGT---GGTCGAAATTTACC 506
maltase.Am.nuc TTATTACATATGGGTTGATCCTGTCAAAGACGATAAAGGAAATCCAATTAAAGACAAATA 500
ScrA.Ls.nuc    GTTTCGTAATTGCCTTTGTGGGGACATACTTATACGGTAAAAAGGCAATGAAGACAACTG 537
                  *      **         *  *

AG.Af.nuc      ACCAAATAATTGGGTAGGCGTATTTGGTGGATCAGCTTGGTCATGGCGGGAAGAACGACA 559
AG.Am.nuc      ACCAACTAATTGGGTAGGCGTGTTTGGTGGATCAGCTTGGTCGTGGCGGGAAGAACGACA 559
AG.Dm.nuc      GCCCAACAATTGGCTGTCGGTGTTCTCCGGATCCGCTTGGATGTGGAACGATGAGAGGCA 559
maltase.Cs.nuc CCCAACTAATTGGGTAAGTGCCTTCAGAAGTAGTGCCTGGGAATGGAACGAAGAACGTGG 566
maltase.Am.nuc TCCTAATAATTGGCTTAGTGTATTCAATGGTACAGGATGGACTTTCCACGAGGGTAGGAA 560
ScrA.Ls.nuc    AAGAAGAAATAATCAATGAAGCACCAGCTACCCCAGA-AGTAGTGGAGAGATTACAAGAT 596
                  *   ***                         *    *      **

AG.Af.nuc      GGCATATTATCTGCACCAATTTGCACCAGAACAACCAGATTTAAATTACTA--TAATCCA 617
AG.Am.nuc      GGCATATTATCTGCATCAATTTGCACCAGAACAACCAGATCTAAATTACTA--TAATCCA 617
AG.Dm.nuc      GCAGTACTATCTCAGGCAGTTCACTTATGGACAACCCGATTTGAACTACCG--AAATCCC 617
maltase.Cs.nuc CGAATATTATTTACATCAATTTTTGGCACAACAACCCGATTTGAATTACCG--CAATCCA 624
maltase.Am.nuc ACAATTTTATTTCCATCAATTTTATAAGCAACAACCAGACTTGAACTACAG--AAACTCG 618
ScrA.Ls.nuc    GAAAAGATTAGTGCACCAGTTACCGGACGAATTGTTGACTTAGCATCAGTACCTGATCCA 656
                  *   *     ** **              *             *  *     *    *

AG.Af.nuc      GCT---GTACTGGATGAAATGC-AAAACGTTCTTAGATTCTGGTTGAA-GAGAGGACTTG 672
AG.Am.nuc      GTT---GTACTGGATGATATGC-AAAATGTTCTCAGATTCTGGCTGAG-AAGGGGATTTG 672
AG.Dm.nuc      GCC---GTGATTAAGGCCATGG-ATGATGTGATGCTCTTCTGGCTAAA-CAAGGGTATTG 672
maltase.Cs.nuc AAA---GTGGTTGAAACAATGA-AAAACGTTTTAAGATTCTGGCTTAG-CAAAGGTATCA 679
maltase.Am.nuc GAT---GTGAGAGAAGAGATGA-AGAATATAATGAAATTTTGGTTGGA-TAAAGGAATCG 673
ScrA.Ls.nuc    GTTTTTGCAAGTGAAGCAATGGGAAAAAGGCATTGCGATTATGCCAACTTCTCAGGATGTA 716
                  *       *       ***  *   *    *    ** **        **

AG.Af.nuc      ATCGTTTCAGA-GTAGATGCTCTGCCTTAC--ATTTGCGAAGATATGCGAT-TCTTAGAC 728
AG.Am.nuc      ATCGTTTCAGA-GTAGATGCTCTGCCTTAC--ATTTGCGAAGACATGCGAT-TCTTAGAC 728
AG.Dm.nuc      CCCGCTTCCGC-ATCGATGC--CATTATATATATTTACGAGGATGCTCAAC-TGAGGGAT 728
maltase.Cs.nuc ATCGATTCAGA-ATTGATGCGGTACCATATTTGTTTGAAGTGGGACCAGATGCGAATGGA 738
maltase.Am.nuc ATCGATTCCGC-ATAGATGCTGTACCACATTTATTCGAAAGCGCTAACATATCGTTAGAT 732
ScrA.Ls.nuc    CTTGCACCAGTTACCGGTGTGATAACAATTGCGGCTAATACTGGTCACGCA-TACGGGAT 775
                  *   *  *     *  **                              *

AG.Af.nuc      GAACCCCTATCAGG---TGAAACAAATG---ATCCC-AACAA---AACTGAG---TACAC 775
AG.Am.nuc      GAACCTCTATCAGG---TGAAACAAATG---ATCCC-AATAA---AACCGAG---TACAC 775
AG.Dm.nuc      GACCCTCCGAGTGGCACT---ACCGATG---ATCCA-AATAATGAGGCC------TACTT 775
maltase.Cs.nuc AATTATCCAGATGAAATTGAAACCCATGCATGCTCA-GATCCTTTATCTCAATGTTACTT 797
maltase.Am.nuc GAACCACCTTTGGG---TAAAAATCTCA---ACTTA-AGTCTCCACGCT------TCTTT 779
ScrA.Ls.nuc    AAAATCGGATGATGGTGCAGAAGTGCTA---ATTCATATTGGTTTAGATACAGTTAATTT 832
                  *                    *

AG.Af.nuc      TCTCAAGATCTACACTCACGATAT-CCCAGAAACCTACAATGTAGTT------------- 821
AG.Am.nuc      TCTCAAGATCTACACTCACGATAT-CCCAGAAACCTACAATGTAGTT------------ 821
AG.Dm.nuc      GACCCACATCTATACCAGAAATCA-GCCTGAGGATTACGGTCTACTT-CAGC----ATTG 829
maltase.Cs.nuc GTTTCACGATTACACTCAAAACAG-GCCTGAAACTTTTGAAATGGTCACGGA----ATGG 852
maltase.Am.nuc AAFTCACACTTTAACGAAAGATCA-ACCCGAGACTTACGAATTGGTAAAAGA----AT-G 833
ScrA.Ls.nuc    AAATGGTATAGGTTTTGAAAAGATTGTCCAACAGGGACAACATGTTAGCGAAGGCGATTT 892
                  *       *   *           *  *

AG.Af.nuc      --CGCAAATTTAGAGATGTGTTAGACGAATTCCCG-----CAACCAAAACACATGCTTA- 873
AG.Am.nuc      --CGCAAATTTAGAGATGTGTTAGACGAATTCCCG-----CAACCAAAACACATGCTTA- 873
AG.Dm.nuc      GCCGCAACTTCTGGATAATTATACAGCTAACCACGATGGGCCATTGAGGATAATGATGA- 888
maltase.Cs.nuc AGFGCGACTTTGGAGGAATT-TAAACAAAAGAATGGAGGACCAACAAGAGTTTTAATGG- 910
maltase.Am.nuc GCCAGATTTGTGTGGACAACTATGCAGAAGAAATAAGCGGGATGAAATAGTACTTTGA- 892
ScrA.Ls.nuc    ATTAGGTCATTTTGATATTGATAAGATTAAACAAGCCGGGCTAACACCGCTAACAATGAC 952
                  *             *                        *                *

AG.Af.nuc      --TCGAGGCATACACGAA----TTTGTCCATGACGATGAAATATTACGAT---------- 917
AG.Am.nuc      --TCGAGGCATACACGAA----TTTATCGATGACGATGAAATATTACGAT---------- 917
AG.Dm.nuc      --CCGAGGGTTATGCTTC----GGTGTCGCAACTAATGGAATACTATGAA-GATTCGAAT 941
maltase.Cs.nuc --TAGAAGCTTATGCTCC----ATTAACAAAAGTAATTCAAATTTATGGTCAAAATGGAC 964
maltase.Am.nuc --CAGAGGCGTATTCTTC----TTTAGAGAACACTCTCAAATATTACGAA--------- 936
ScrA.Ls.nuc    TATTGTGACGAATACAGCGGGATATGCACAAGTTGATCCGCTTTTAACAGTCGACAAGGC 1012
                  *        *  *         *         *         *   **
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Figure 4.5 (continued)

```
AG.Af.nuc      ------TACGGAGCAGATTTTCCCTTCAATTTTGCATTCATCAAGA--ACGTCTCTAGGA  9C9
AG.Am.nuc      ------TACGGAGCAGATTTTCCCTTCAATTTTGCATTCATCAAGA--ATGTTTCTAGGG  9C9
AG.Dm.nuc      GGTGTACAGGGCCCCCAGTTTCCCTTCAACTTTGACTTCATCACCG--AACTGAATGCCA  9C9
maltase.Cs.nuc AGC-TAAATGGAGCTCAAATTCCATTTAATTTCGAGTTCTTGAATA--ATTTGGGAGCCG  1C21
maltase.Am.nuc ------GTTGGTTCAAATGTTCCCTTCAATTTTAAATTTATAACAG--ATGCAAATTCAT  9E8
ScrA.Ls.nuc    TGC-TATGCAAGGCGAAGAAATTATTCAATTACACGCTAAAAAGGATTAAGGGGTAGTTA  1C7L
                   *  *         ** ** *        *     *        *

AG.Af.nuc      ATTCAAATTCATCAGACTTCAAAA---AATTGGTCGATAATTGGATGACGTACATGCCAC  1C26
AG.Am.nuc      ATTCAAATTCATCAGACTTCAAAA---AATTGGTCGATAATTGGATGACGTACATGCCAC  1C26
AG.Dm.nuc      ATTCGACAGCTGCGGACTTTGTCT---TCTATATCTCCAGGTGGCTCATCTATATGCCAC  1C56
maltase.Cs.nuc TAAGTAATGCTCGTGATTTCAAAG---ACGTAATTGACAATTATCTCAGCACAATCCCAG  1C78
maltase.Am.nuc CTTCCACGCCAGAACAATTTAAAG---TAATTATAGACAATTGGATAAAAGGAACGCCCC  1C45
ScrA.Ls.nuc    CATGCAAACTAACTGGTGGCAAAATGCAGTATTTTATCAAGTCTATCCAAGAAGT-TTTC  1130
                   *                            *    *   *    *

AG.Af.nuc      CAANTGGTATTCCTAACTGGGTGCCCGGAAACCANGACCNATTGAGATNGGTGTCGAGAT  1C86
AG.Am.nuc      CAAGTGGTATTCCTAACTGGGTGCCCGGAAATCACGATCAATTGAGATTGGTGTCGAGAT  1C86
AG.Dm.nuc      ATGGTCATGTGGCCAACTGGGTGATGGGAAATCACGACAATCCTCGAGTGGCATCACGAT  1116
maltase.Cs.nuc AAGGAGCAACACCAAATTGGGTTCAAGGAAATCACGATCAACATCGATCAGCATCACGAC  1133
maltase.Am.nuc AAAATAATGTTCCAAATTGGGTGATGGGAAACCATGATCGAGTTCGTGTCGGTACACGTT  11C5
ScrA.Ls.nuc    AAGATAGTAATGGAGATGGAATTGGTGATATTCAAGGTATTATTCAAAGATTAGATTACC  1F9C
                   *    *    *     *   *    *  ** *

AG.Af.nuc      TTGGAGAAGAGAAGGGCCGTATGATCACCACGATGTCGCTTTTGC---TGCCAGGTGTTT  1143
AG.Am.nuc      TTGGAGAGGAGAAGGCCCGTATGATCACCACGATGTCGCTTTTGC---TGCCAGGTGTTG  1143
AG.Dm.nuc      TCGGTGAGAAATCTGTGGACGCCATGAATATGCTGCTGATGACAT---TGCCAGGAATTG  1173
maltase.Cs.nuc TCGGTCCACAAAAAGCTGATGCAGTTAATATGTTACTTCAAGTTC---TTCCCGGAGCTG  1195
maltase.Am.nuc ATCCTGGTAGGGCGGATCACATGATAA---TGTTGGAGATGATTT---TGCCTGGAGTCG  1159
ScrA.Ls.nuc    TAGCTGATCTGGGTGTAAATGCAATTTGGCTATCACCAGTTTATCAATCCCCTAATGTTG  12EC
                          *          *                        **

AG.Af.nuc      CCGTGAATTATTACGGTGATGA--AATTGGTA--TGTC---GGATACTTATATCTCGTGG  1195
AG.Am.nuc      CCGTGAATTACTACGGTGATGA--AATTGGTA--TGTC---GGATACTTATATCTCGTGG  1195
AG.Dm.nuc      GTATTACTTATAATGGCGAGGA--GTTGGGCA--TGACTGACTACAGGGACATCAGCTGG  1229
maltase.Cs.nuc CAGTCACTTATTATGGTGAAGA--ACTTGCAA--TG---GAAGACGTTTTCGTTCCATGG  1243
maltase.Am.nuc CGGTCACGTATTATGGAGAAGA--AATCGGTA--TGGT-------------------G  1194
ScrA.Ls.nuc    ATAATGGCTATGATATTTCAGATTATCAGGCAATTAATCCGGAATATGGTTCTATGGTGG  121C
                   ** *       **     * *  *

AG.Af.nuc      G-AGGACACGCAGGATCCACAGGGATGCGGTGCCGGTAAAGAAAACTATCAAACAAT-GT  1254
AG.Am.nuc      G-AGGATACGCAGGATCCGCAGGGATGCGGCGCCGGTAAAGAAAACTATCAAACGAT-GT  1254
AG.Dm.nuc      A-GCGATACGGTGGATCAGCCCGCTTGTGAGGCTGGAATCGACAACTACAAGACGAT-TT  1287
maltase.Cs.nuc T-CTCGTACTGTCGATCCACAAGCATGTACAACAGATCCAAATATTTTCCATGCCAA-GT  13C5
maltase.Am.nuc G-ATAACACT-ACGATATATAAATATGATGTAC-----GTGATGGTTGTCGTACACC-AT  1245
ScrA.Ls.nuc    ATATGGAGCAGTTAATTGAAGCGGCGAAGATTCGTAAGATTAAAATTGTTATGGACTTAG  137C
                   *     **            *         *         *      *

AG.Af.nuc      CGAGAGATCCCGCGAG-----AACGCCATTCCAATGGGACGACTCACTTTCTGCTGGATT  1309
AG.Am.nuc      CGAGAGATCCCGCGAG-----AACGCCATTCCAATGGGACGACTCAGTTTCTGCTGGATT  1309
AG.Dm.nuc      CTAGAGATCCTGAGCG-----AACTCCCATGCAATGGAGTAGTGATGTGAATGCAGGATT  1342
maltase.Cs.nuc CACGTGATCCCGCAAG-----AACACCCATGATTTGGACTTCACAAAAAAACGCAGGATT  1361
maltase.Am.nuc TCCAA-----TGGGAT-----AACTCCATTAATGCAGGCTTTAGTAA-AATCGCTGAAAA  1295
ScrA.Ls.nuc    TTGTTAATCATACAAGTGACCAACATCCATGGTTTTTAGAAGCACGAAAATCAAAAGATA  143C
                   ***  *  *                                     *

AG.Af.nuc      TTCC----TCAAGCTCTAATACGTGGCTTCGTGTCAATGAAAATTACAAGAC---TGTCA  1362
AG.Am.nuc      TTCC----TCAAGCTCTAATACCTGGCTTCGTGTCAACGAAAATTACAAGAC---TGTCA  1362
AG.Dm.nuc      CTCC----TCCGCCGATCGCACTTGGTTGCCTGTCAATCCGAATTATAAGGA---ACTTA  1395
maltase.Cs.nuc TTCA----AGTTCAAATTACACATGGCTTCCAACTGGACCAGATTATCGCAA---AAATA  1414
maltase.Am.nuc TTTG----CTTGAAAAGAAT---TGGCTACCTGTTCATACATCGTACAAAAGTGGACTAA  1343
ScrA.Ls.nuc    ATCCGTATCGTGATTTTTATATTTGGCGAGACCCTGCAACCGATGGTAGTGTTCCGAATG  149C
                   *                        ***

AG.Af.nuc      ATCTAGCTGCTGAAA--AGAAGGACAA---GAACTCGTTCTTCAATATGTACAAGAAATT  1417
AG.Am.nuc      ATCTAGCTGCTGAAA--AGAAGGACAA---GAACTCGTTCTTCAATATGTTCAAGAAATT  1417
AG.Dm.nuc      ATCTTCGGAATCAGC--AGCAGGCGAG---GCGAAGTCATTACAAGATCTATCAGTCCCT  145C
maltase.Cs.nuc ATGTTGAAGTGCAGC--GTAGTCAGAG---AGGCAGTCACTTGAATATCTTTAAAAAGTT  1469
maltase.Am.nuc ATTTGGAGCAAGAGA--AAAAAGATAG---TATTTCTCATTATCATCTTTATACCAACTT  14J3
ScrA.Ls.nuc    ATTTACAAAGTAATTTTAAAGGATCAGCTTGGGCGTTTGATGCGGTTACTGGGCAATATT  155C
                   ** *      *         *          *       *     *    *      *
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Figure 4.5 (continued)

```
AG.Af.nuc      CGCGTTGCT-------GAAAAAATCGCCATATTTTAAAGAGGCCAATTTAAGTACGAGGA 1470
AG.Am.nuc      TGCGTCGCT-------GAAAAAATCGCCATACTTTAAAGAGGCCAATTTAAATACGAGGA 1470
AG.Dm.nuc      TCTGAAGCT-------CAGACAACTGCCAGTTCTGAAGAACGGATCCTTTGTTCCAGAAG 1503
maltase.Cs.nuc GACTCAACT-------TCGTAAGCAAGACATTTTGATGTATGGCACTTATGATAGTTACT 1522
maltase.Am.nuc GACCGCTTT-------AAGAAAGAGAGATGTGTTGAAAAAAGGAAACTTTACTATAGAAA 1456
ScrA.Ls.nuc    ATTTACATTTTTATGCGAAAGAACAACCGGATTTAAATTGGCAAAATCCTAAAGTTAGAG 1610
                      *           *          *  *

AG.Af.nuc      TGCTGAACGACAATGTTTTCGCNTTCTCTAGG-GAAACCGAAGACAATGGATCTCTTTAC 1529
AG.Am.nuc      TGCTGAACGACAATGTTTTCGCATTCTCTAGG-GAAACCGAAGATAATGGATCTCTTTAC 1529
AG.Dm.nuc      TGGTTAATCGCAGGGTCTTCGCTTTCAAGCGA-GAACTGAAGAACGAGCACACTCTGCTG 1562
maltase.Cs.nuc TGGCAAATGATGACGTTTTGGTGATTAAACGT-GAAATTGAGAATAATCGAACTTTGATT 1581
maltase.Am.nuc TTTTAAACAAAACTGTTCTGGCTGTCGTGCGACAAAGCGAAGAAGAAGCGGTATCTCTTT 1516
ScrA.Ls.nuc    AAGCTGTCTACCAGATGATGACTTGGTGGCTT-CAAAAAGGGATTGGTGGTTTTAGGATG 1669
                      *  *                       **                    *

AG.Af.nuc      GTAATAATGAACTTCTCGAA---CGAGGAACAAATCGTGGATTTGAAAGCGTTTG----- 1581
AG.Am.nuc      GCAATATTGAACTTCTCGAA---CGAGGAACAAATCGTGGATTTGAAAGCGTTCA----- 1581
AG.Dm.nuc      ACCATTGTGAACGTGAGCAACCGCACTGAACTGGTTGACATCGCGGACTTTA-------- 1614
maltase.Cs.nuc GCTGTCCTTAACTTGGGT---TTCACTGAACAAGTCGTCAATTTGAATTTAAATGACCGA 1638
maltase.Am.nuc ----TGATCAACTTCTCTAAAAATAATACTATCGTGGATATATCAAAGTTGGT------- 1565
ScrA.Ls.nuc    GACGTTATTGATTTGATAGG--GAAGGAACCTGACCGCAAAATTAAGGAAAACGGACCGC 1727
                  *   *   *   *                               *

AG.Af.nuc      ----ATC-----ACGTGCCGAAGA-GATTGAATATGTTTTACAACAATTTTAACTC---- 1627
AG.Am.nuc      ----ATA-----ACGTGCCGAAAA-AATTGAATATGTTTTACAACAATTTTAACTC---- 1627
AG.Dm.nuc      ---TAGA-----ACAGCCCA-ATC-GATTGAGTGTCCTTGTGGCGGGAGTGGACTCGCAA 1664
maltase.Cs.nuc GATTGGA-----AAGTTCCAGAGA-GAATGGAAGTTGCAACAGCTTCAGTTAACGCAGGA 1692
maltase.Am.nuc -----GA-----ACAAAGAAATA-ATGCTAAAATTTACACAAGCAGCGTAAACTCCAA- 1613
ScrA.Ls.nuc    AATTACATGCGTATCTTCAAGAGATGAACGCAAGGGTACTTTCACAGTATGATGTAGTAA 1787
                   *         *                              *

AG.Af.nuc      ----TGATATAAAATCCATCTCCAACAACGAGAAAATAAA-AGTTCCTGCTTTAAGATTT 1682
AG.Am.nuc      ----TGATATAAAGTCCATCTCCAACAATGAACAAGTAAA-AGTTTCTGCTTTAGGATTT 1682
AG.Dm.nuc      CACCGGGTGGGGGATCGACTTAAGGCCGAGACAATTGAATTGGCGCCCAACGAGGGATTA 1724
maltase.Cs.nuc ATGTTCGAGAGACAACCCGTTGTGACAAGTGAAGTCTACGTATCAGCTGGCGTTGGAGTT 1752
maltase.Am.nuc --TTTGACAGTAAATCAAACTGTAAATCCAGTGGCTATCAATATTCCTGGAGATACATCT 1671
ScrA.Ls.nuc    CGGTTGGAGAGACAT-GGGGGGCAACACCCGAAATTGGCCAGATG--TACAGTAATCCTA 1844

AG.Af.nuc      NTAATCTTAATCTCTCA--AGATGCTAAATTTGAAAACATTTAATTTCTTCTTGAACATG 1740
AG.Am.nuc      TTCATCTTAATTTCTCA--AGATGCTAAATTTGGAAACTTTTAATTTCTTCCTGAATATG 1740
AG.Dm.nuc      GTTATTCAGCTGAATAAGCGAAAGTAA--------------------------------- 1751
maltase.Cs.nuc GTT-CTCGATTATCAAGTAGGGCGTCAAATTCCCGAACCAAGAGGTGACGATCCAGGACT 1811
maltase.Am.nuc ATAATTGTAGATTCATC---CACTTCAGGCGCTACTATAGTCAATTATTCAATCATGAT- 1727
ScrA.Ls.nuc    ATCGCCACGAACTATCGATGATCTTTCAATTTGAACAAATTAATTTAGATAAACAATCAG 1904
                  *

AG.Af.nuc      TCTATNCTTTGAAGCGGCGA--------------------- 1760
AG.Am.nuc      TCTATTCTTTGAAGCGGCGA--------------------- 1760
AG.Dm.nuc      ----------------------------------------- 
maltase.Cs.nuc ATACGAATAAGAAATATTCC--------------------- 1831
maltase.Am.nuc TTTCTTATCCGCAGTGTTCATATCTTTTTTCCAACGG----- 1764
ScrA.Ls.nuc    GGATGACTCGCTGGGATTTAAAACCACTTATTCCAGCAGAGT 1946
```

Figure 4.5 The multiple alignment of nucleotide sequences of *AG* in *A. florea* with other organisms. Common residues are indicated by asterisks below the sequences.

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              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  10         20         30         40         50         60         70
AG.Af.nuc     ---MKAVIVF CLMALSIVDA AWKPLPEN-- --LKEDLILY QVYPRSFKDS NGDGIGDIIG IKEKLDHFLE
AG.Am.nuc     ---MKAVIVF CLMALSIVDA AWKPLPEN-- --LKEDLIVY QVYPRSFKDS NGDGIGDIEG IKEKLDHFLE
AG.Dm.nuc     MVVVKIAFIL SVGLVGILAH KHQSKELDAK YNWWQHEVFY QIYPRSFQDS NGDGIGDLQG ITSRLQYFKD
maltase Cs.   MIPFKKLTIL LSIACSVLAA PEGAREKE-- --WWEIGNFY QVYPRSFMDS DGDGVGDLKG ISEKVGYLKE
maltase Am.   ---MKSLVVV VLLLAVGLGA GQN--NKG-- --WWKNAIFY QVYPRSFMDS NSDGIGDLKG IKDKLSHFIE
ScrA.Ls.nu    ----FVPLIP ALTAGGLLMA INNVLTGQG- --LFGAQSIV QMFP------ ------QWKG FAEIVNMMSS
Clustal Co           :          :                  . *::*          : * : .: : .


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  80         90        100        110        120        130        140
AG.Af.nuc     MGVDMFWLSP IYPSPMVDFG YDISNYTDWH PIFGTISDLD DLVSAAHEKG LKIILDFVPN HTSDQHKWFQ
AG.Am.nuc     MGVDMFWLSP IYPSPMVDFG YDISNYTDWH PIFGTISDLD NLVSAAHEKG LKIILDFVPN HTSDQHEWFQ
AG.Dm.nuc     TGITSVWLSP IYESPMVDFG YDISNYTNEQ PEYGTLEDFD ALIAKANELG VKVILDFVPN HSSNKHPWFI
maltase Cs.   IGMDGVWLSP IFDSPMADFG YDISNFTKWF PQFGDLSSID ELVAEFNKKD MKLILDFVPN HTSDQCEWFK
maltase Am.   SGITAIWLSP INRSPMVDFG YDISDFKDVD PIFGTIKDLE DLTAEAKKQN LKVILDLVPN HTSDQHKWFQ
ScrA.Ls.nu    APFTFLP--- ------ILIA FSATKRFGGN PYLGAAAGMM LVMPNLVN-G YGVAESIATG HMT---YWHV
Clustal Co        . .                 :. :. :.    *  *   .:   :  :        :    .:.... * :    *.


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  150        160        170        180        190        200        210
AG.Af.nuc     LSLK-----N VEPYNNYYIW HPG-KIVNGK R---VPPNNW VGVFGGSAWS WREERQAYYL HQFAPEQPDL
AG.Am.nuc     LSLK-----N IEPYNNYYIW HPG-KIVNGK R---VPPTNW VGVFGGSAWS WREERQAYYL HQFAPEQPDL
AG.Dm.nuc     KSVA-----R EPGYEDFYVW EDGILLENGT R---VPPNNW LSVFSGSAWM WNDERQQYYL RQFTYGQPDL
maltase Cs.   KSIQ-----R DPEYNDYYIW HPGKPNPDGG RN--LPPTNW VSAFRSSAWE WNEERGEYYL HQFLAQQPDL
maltase Am.   MSINNTNNNN TNKYKDYYIW VDPVKDDKGN PIKDKYPNNW LSVFNGTGWT FHEGRKQFYF HQFYKQQPDL
ScrA.Ls.nu    FGLN----IA QAGYQGQVIP VIGVAFILAN LE--KFFHKH LNDAVDFTFT PMLSIIITGF LTFTLVGPAL
Clustal Co        .:          *:. :        .         : :.  :     :     * * *


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  220        230        240        250        260        270        280
AG.Af.nuc     NYYNPAVLDE MQNVLRFWLK RGLDGFRVDA LPYICED--- --MRFLDEPL SGETNDPNKT EYTLKIYTHD
AG.Am.nuc     NYYNPAVLDD MQNVLRFWLR RGFDGFRVDA LPYICED--- --MRFLDEPL SGETNDPNKT EYTLKIYTHD
AG.Dm.nuc     NYRNPAVIKA MDDVMLFWLN KGIAGFRIDA IIYIYED--- --AQLRDEPP SGTTDDPNNE AYLSHIYTRN
maltase Cs.   NYRNPKVVET MKNVLRFWLS KGINGFRIDA VPYLFEVGPD ANGNYPDEIE THACSDPLSQ CYLYHDYTQN
maltase Am.   NYRNSDVREE MKNIMKFWLD KGIDGFRIDA VPHLFES--- -ANISLDEPP LGKNLNLSLH ASLNHTLTKD
ScrA.Ls.nu    RIVSNGVTDS LVWAYQTLGA VGMGIFGLGY SAIVLTG--- ----LHQSFP AIETTLLADI AKTGGSFIFP
Clustal Co    . . * . :        *: * :.            :.


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  290        300        310        320        330        340        350
AG.Af.nuc     IPETYNVVRK FRDVLDEFPQ -----PKHEL IEAYTNLSMT MKYYD----- YGADFPFNFA FIKNVSRNSN
AG.Am.nuc     IPETYNVVRK FRDVLDEFPQ -----PKHEL IEAYTNLSMT MKYYD----- YGADFPFNFA FIKNVSRDSN
AG.Dm.nuc     QPEDYGLLQH WRQLLDNYTA NHDGPLRIEM TEGYASVSQL MEYYEDSNGV QGPQFPFNFD FITELNANST
maltase Cs.   RPETFEMVTE WRATLEEFKQ KNGGPTRVLM VEAYAPLTKV IQIYGQNGQL NGAQIPFNFE FLNNLGAVSN
maltase Am.   QPETYELVKE WRDFVDNYAE ENKRDEIVLL TEAYSSLENT LKYYE----- VGSNVPFNFK FITDANSSST
ScrA.Ls.nu    VAAMANIAQG AATFAVFFVT KN---KQQFS LTTSAGISAM LGITEP--AL FGVNLKLKFP FFIGLIASGI
Clustal Co        .   :         :             : :      :      * :. ::*  *:    .


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  360        370        380        390        400        410        420
AG.Af.nuc     SSDFKKLVDN WMTYMPPXGI PNWVPGNXDX LRXVSRFGEE KGRMITTMSL LLPGVSVNYY GDEIGMSDT-
AG.Am.nuc     SSDFKKLVDN WMTYMPPSGI PNWVPGNHDQ LRLVSRFGEE KARMITTMSL LLPGVAVNYY GDEIGMSDT-
AG.Dm.nuc     AADFVFYISR WLIYMPHGHV ANWVMGNHEN PRVASRFGEE SVDAMNMLLM TLPGIGITYN GEELGMTDYR
maltase Cs.   ARDFKDVIDN YLSTIPEGAT PNWVQGNHEQ HRSASRLGPQ KADAVNMLLQ VLPGAAVTYY GEELAMEDV-
maltase Am.   PEQFKVIIDN WIKGTPQNNV PNWVMGNHDR VRVGTRY-PG RADHMIMLEM ILPGVAVTYY GEEIGMVDN-
ScrA.Ls.nu    SSFIIGLLHV LSVSMGPAGI IGFIAIAPKS IP-------- SFMMGAIISF VIAFVGTYLY GKKAMKTTEE
Clustal Co    . :  :        .::        .          :     :. .   *.:


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  430        440        450        460        470        480        490
AG.Af.nuc     YISWEDTQDP QGCGAGKENY QTMSRDPART PFQWDDSLSA GFSSSSN--- --TWLRVNEN YKT-VNLAAE
AG.Am.nuc     YISWEDTQDP QGCGAGKENY QTMSRDPART PFQWDDSVSA GFSSSSN--- --TWLRVNEN YKT-VNLAAE
AG.Dm.nuc     DISWSDTVDQ PACEAGIDNY KTISRDPERT PMQWSSDVNA GFSSADR--- --TWLPVNPN YKE-LNLRNQ
maltase Cs.   FVPWSRTVDP QACTTDPNIF HAKSRDPART PMIWTSQKNA GFSSSNY--- --TWLPTGPD YRK-NNVEVQ
maltase Am.   ---------- ------TTIY KYDVRDGCRT PFQWDNSINA GFSKIAENLL EKNWLPVHTS YKSGLNLEQE
ScrA.Ls.nu    EIINEAPATP EVVERLQDEK ISAPVTG--R IVDLASVPDP VFASEAMG-- --KGIAIMPT SQDVLAPVTG
Clustal Co                                  . . .. *:.          . :           :


              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  500        510        520        530        540        550        560
AG.Af.nuc     KKDKNSFFNM YKKFALLKKS PYFKEANLST RMLNDNVFAF SRETEDNGSL YVIMNFSNEE QIVDLKAFDH
AG.Am.nuc     KKDKNSFFNM FKKFASLKKS PYFKEANLST RMLNDNVFAF SRETEDNGSL YAILNFSNEE QIVDLKAFNN
AG.Dm.nuc     QQARRSHYKI YQSLLKLRQL PVLKNGSFJP EVVNRRVFAF KRELKNEHTL LTIVNVSNRT ELVDIADFIE
maltase Cs.   RSQRGSHLNI FKKLTQLRKQ DILMYGTYDS YLANDDVLVI KREIENNRTL IAVLNLGFTE QVVNLNLNDR
maltase Am.   KKDSISHYHL YTNLTALRKR DVLKKGNFTI EILNKTVLAV VRQSEEE-AV SLLINFSKNN TIVDISKLVN
ScrA.Ls.nu    VITIAANTGH AYGIKSDDGA EVLIHIGLDT VNLNGIGFEK IVQQGQHVSE GDLLGHFDID KIKQAGLTPL
Clustal Co        :            :              :      *   : :. :   ::.       :  :
```

Figure 4.6 (continued)

```
        ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|...
            570         580         590         600         610         620
AG.Af.nuc   V---PKRLNM FYNNFNSDIK SIS--NNEKI KVPALRFXIL ISQDAKFENI F--------- --------
AG.Am.nuc   V---PKKLNM FYNNFNSDIK SIS--NNEQV KVSALGFFIL ISQDAKFGNF ---------- --------
AG.Dm.nuc   Q---PNRLSV LVAGVDSQHR VGDRLKAETI ELAPNEGLVI QLNKRK---- ---------- --------
maltase Cs. DWKVPERMEV ATASVNAGMF ERQPVVTSEV YVSAGVGVVL DYQVGRQIPE PRGDDPGLYE --------
maltase Am. K---RNNAKI YTSSVNSNLT VNQTVNPVAI NIPGDTSIIV DSSTSGATIV NYSIMIFLSA VFISFFQR
ScrA.Ls.nu  T-------MT IVTNTAGYAQ VDPLLTVDKA AMQGEEIIQL HAKKD----- ---------- --------
Clustal Co        . .        . .          :        :  .
```

Figure 4.6 The multiple alignment of amino acid sequences deduced from the cDNA sequences of AG in *A. florea* with other organisms. Common residues are indicated by asterisks below the sequences.

The cDNA sequence was blasted and aligned with *AG* in *A. mellifera*. The result of blast showed 95% identity. Furthermore, the cDNA sequence was multiple aligned with sequences of *AG* in *A. mellifera* (D79208), maltase 1 in *A. mellifera* (XM 393379), *AG*, α - amylase, and transporter activity in *Drosophila melanogaster* CG14934 - PA (NM 135678), sucrose - specific enzyme II of the PTS (*ScrA*) and dextran glucosidase (*dexB*) genes in *Lactobacillus sakei* (AF401046), and *Culicoides sonorensis* clone CsMAL1 maltase (AY603565). The similarity between sequences was presented in table 4.2.

Table 4.2 Similarity of the *AG* sequence in *A. florea* (1,739 bp) and that in other organisms.

Organisms	Length (bp)	Score
AG in *A. mellifera*	1,760	95
maltase 1 in *A. mellifera*	1,764	38
AG in *Drosophila melanogaster*	1,751	46
ScrA in *Lactobacillus sakei*	1,946	19
maltase in *Culicoides sonorensis*	1,831	41

A preliminary phylogenetic tree from deduced amino acid of *AG* among these organisms was reconstructed using UPGMA and neighbor-joining (NJ) methods as implemented in the program PAUP* version 4.0b (Phylogenetic Analysis Using Parsimony methods*). To investigate support for nodes estimated in the trees, bootstrap analysis was undertaken in PAUP (heuristic search). The bootstrap analysis with 50% deletion was used as indications of branch support for individual clades. The bootstrap values was calculated by using 1,000 replicates. The *dex*B in *Lactobacillus sakei* sequence was selected as an outgroup in NJ and bootstrap methods. A phylogenetic tree from UPGMA method (Fig. 4.7) was indicated distance between *AG* of *A. florea* among that in other organisms. Furthermore, a phylogenetic tree from NJ method (Fig. 4.8) was represented three major clades. Clade I was comprised of the *AG* of *A. florea* and *A. mellifera*. Clade II was comprised of the *AG* in *Drosophila melanogaster* and maltase in *Culicoides sonorensis*. Clade III was comprised of maltose 1 in *A. mellifera*.

UPGMA
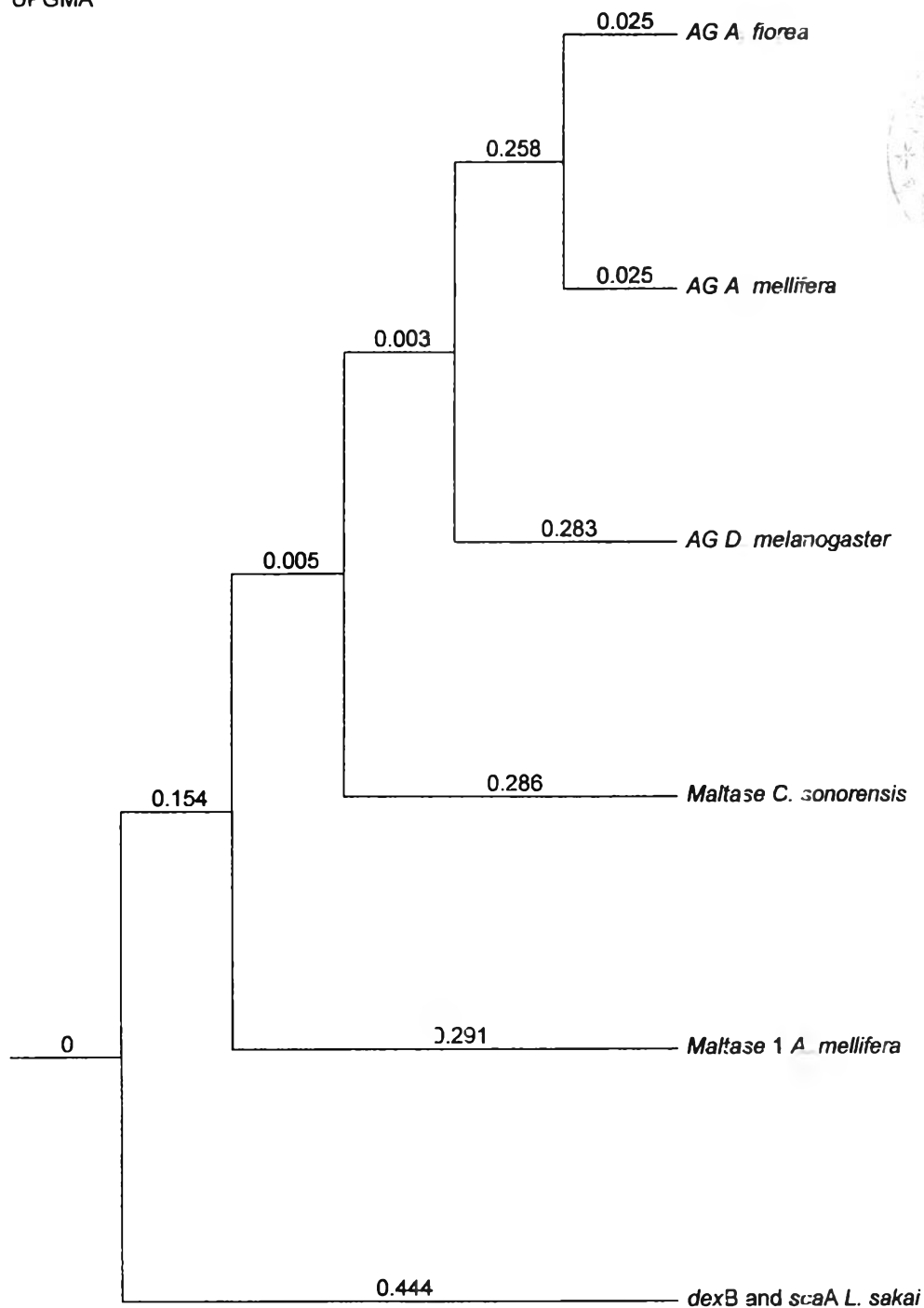
Figure 4.7 A phylogenetic tree of deduced amino acid sequence of *AG* in *A. florea* among other organisms by UPGMA method. A number on each branch indicate differential.

NJ

	0.024	AG A. florea
0.250		
100		
	0.026	AG A. mellifera
0.009		
65		
	0.277	AG D. melanogaster
0.009		
63		
	0.293	Maltase C. sonorensis
0.288		Maltase 1 A. mellifera
0.596		dexB and scaA L. sakai
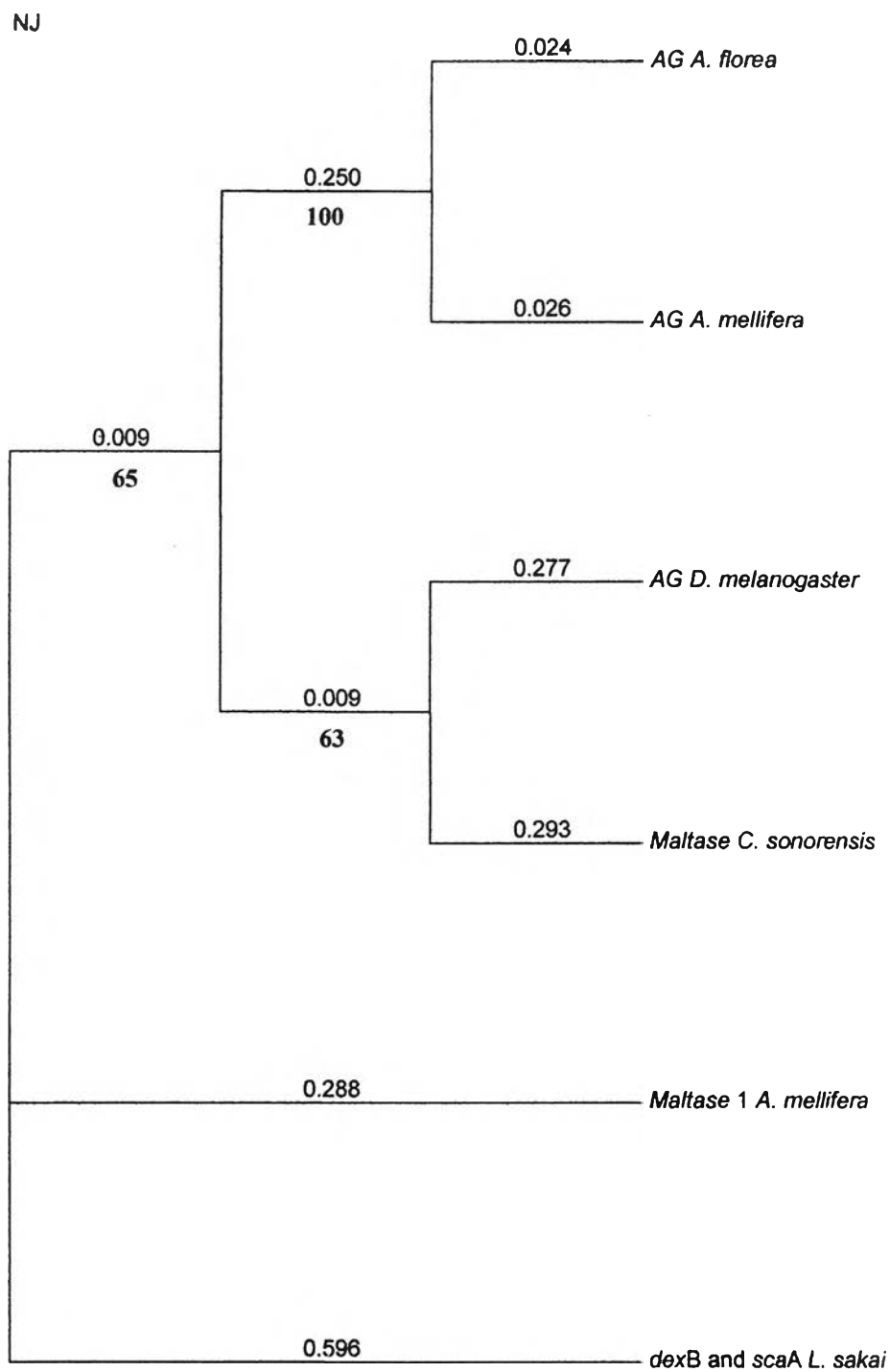
Figure 4.8 A phylogenetic tree of deduced amino acid sequence of *AG* in *A. florea* among that in other organisms by NJ method. The upper numbers on each branch indicate the differential between genes. The lower numbers (in bold type) were the full heuristic bootstrap percentages of 1,000 replicates.

4.3 Major protein pattern of crude extract

Protein of head (12 heads) and honey crop (20 honey crops) of forager bee was extracted by buffer insect saline. Crude protein was separated by SDS - PAGE. Different bands of major protein (50 kDa from head and 15 kDa from honey crop) were observed as in Figure 4.9.
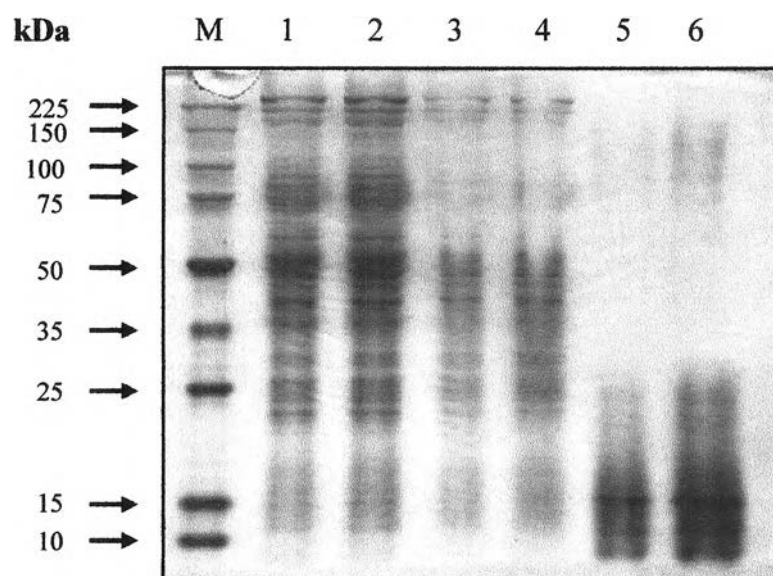
Figure 4.9 Pattern of major proteins in crude of head and honey crop.

Lane M: broad range protein MW markers

Lanes 1 - 2:   crude protein of head (1 mg protein)

Lanes 3 - 4:   crude protein of head (0.5 mg protein)

Lanes 5 - 6:   crude protein of honey crop (1 mg protein)

4.4 Ammonium sulfate precipitation

Various concentrations of ammonium sulfate (AS) were added into crude protein. Due to Fig. 4.10, the highest specific activity (1 u/ mg) was obtained from crude without AS precipitation. High specific activity (0.7 u/ mg) was also from precipitation of 80 - 95% AS. The lowest specific activity (0.2 u/ mg) was appeared from precipitation in 40 - 50% AS. Due to SDS – PAGE, different patterns of protein was observed in each lane. Common band of 100 kDa was observed in all lanes (Fig. 4.11).
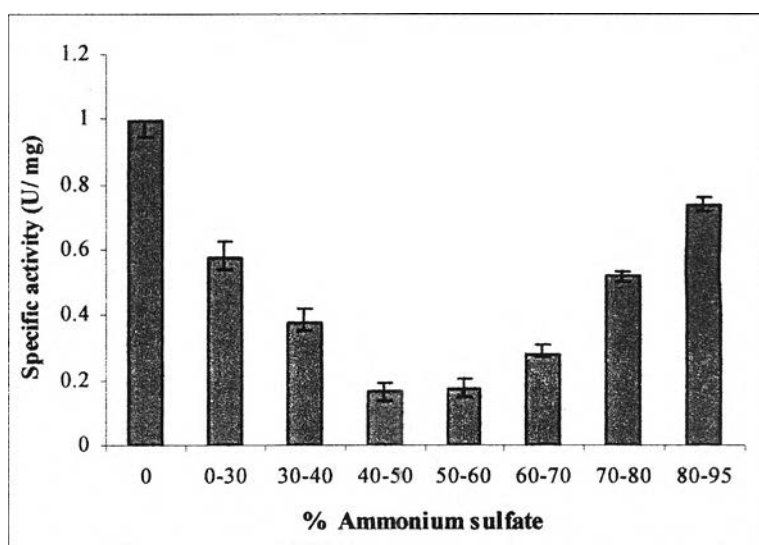
Figure 4.10 Specific activity of crude precipitation by various concentrations of ammonium sulfate.
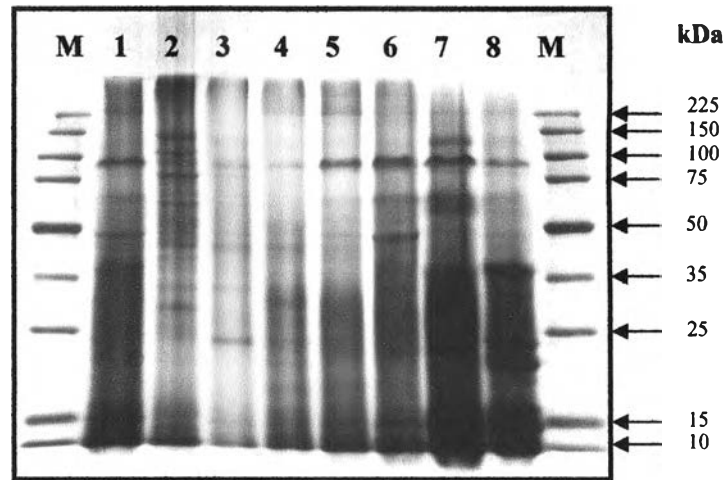
Figure 4.11 Protein profile of precipitate from various concentrations of ammonium sulfate (AS). Protein (20 μg) of all precipitates were electrophoresed by SDS polyacrylamide gel and CBB stained.

Lane 1: precipitate by 0% AS

Lane 2: precipitate by 0 - 30% AS

Lane 3: precipitate by 30 – 40% AS

Lane 4: precipitate by 40 - 50% AS

Lane 5: precipitate by 50 - 60% AS

Lane 6: precipitate by 60 - 70% AS

Lane 7: precipitate by 70 - 80% AS

Lane 8: precipitate by 80 - 90% AS

Lane M: broad range protein MW markers

4.5 AG purification

4.5.1 Crude protein with ammonium sulfate precipitation

4.5.1.1 Anion exchange (DEAE – cellulose)

Crude protein with AS precipitation was injected to DEAE – cellulose equilibrated by 30 mM sodium phosphate buffer (pH 6.3). The column was developed by a linear gradient of 0 – 1.0 M NaCl. SDS – PAGE shown in lanes 1 – 4 in Figure 4.15 (B). The AG activity was eluted within unbound (fraction# 6 – 8) and bound peaks (fraction# 22 – 24) as in Figure 4.12. Positive fractions were pooled and desalted by dialysis with 30 mM sodium phosphate buffer (pH 6.3).
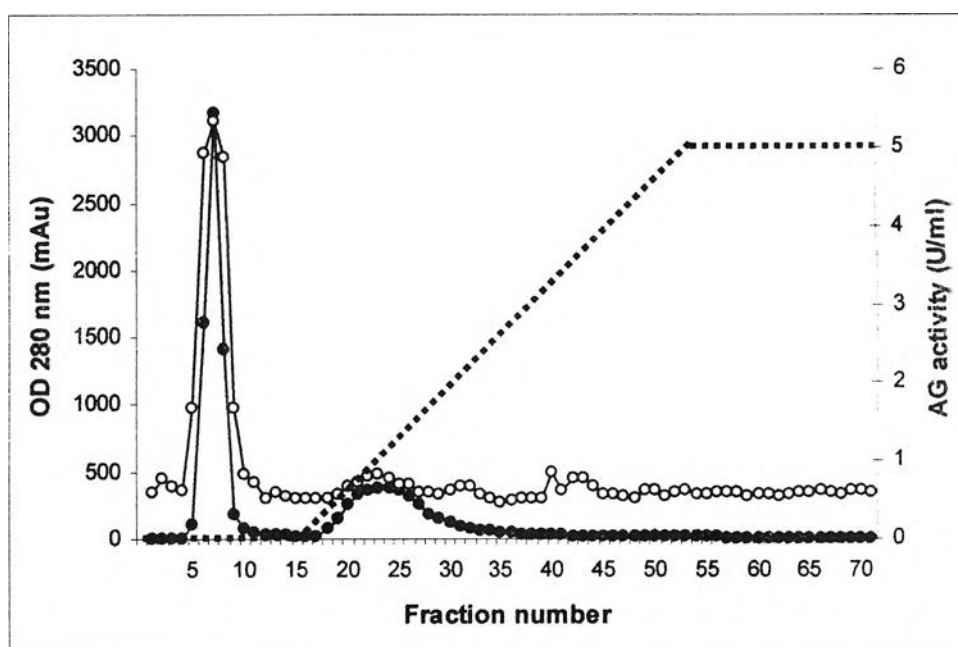
Figure 4.12 AG on DEAE – cellulose. Crude protein, 300 mg; column, 1.6 x 13 cm equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 0 – 1 M NaCl flow rate, 60 ml/ h; fraction size, 10 ml; —●— OD at 280 nm; —○— , AG activity; ········· , molarity of NaCl.

4.5.1.2 Gel filtration (Superdex 200)

The dialyzed sample of bound peak from DEAE – cellulose was applied to a gel filtration column on Superdex 200 equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl (Fig. 4.13). Low AG activity (less than 1 u/ ml) of bound peak sample (fraction# 14) was calculated. SDS – PAGE was shown in lane 1 in Figure 4.15 (A).
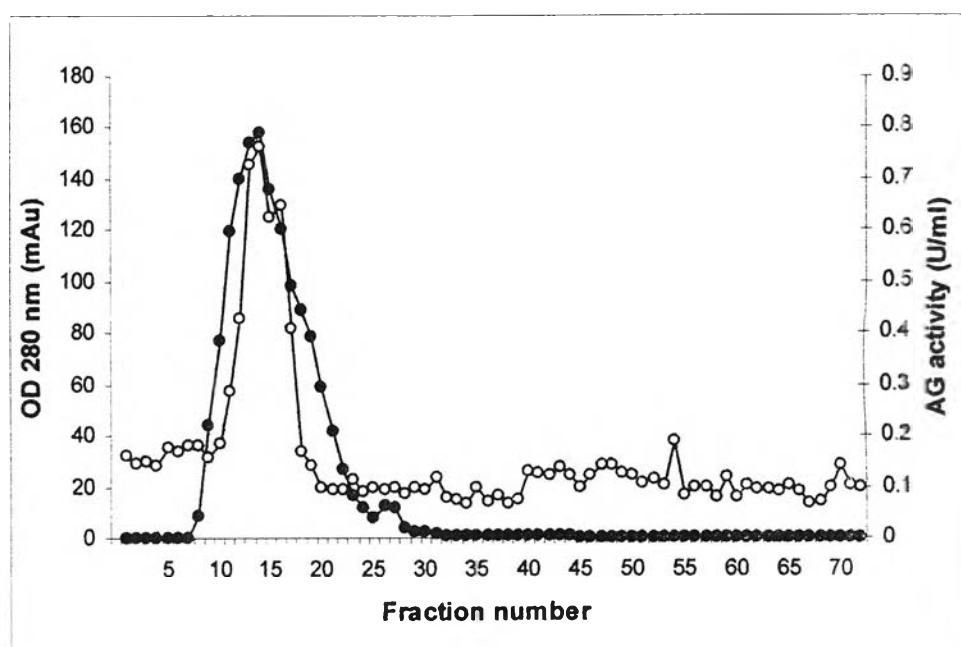
Figure 4.13 AG on a gel filtration (Superdex 200) column. Bound peak sample of DEAE – cellulose, 10 mg protein; column, 1.6 x 51 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/ h; fraction size, 10 ml; —●— , OD at 280 nm; —○— , AG activity.

The dialyzed sample of unbound peak from DEAE – cellulose was applied to a gel filtration column on Superdex 200 with the same condition. High AG activity (4 u/ ml) was obtained as in Figure 4.14. SDS – PAGE was shown in lane 5 in Figu e 4.15 (B).
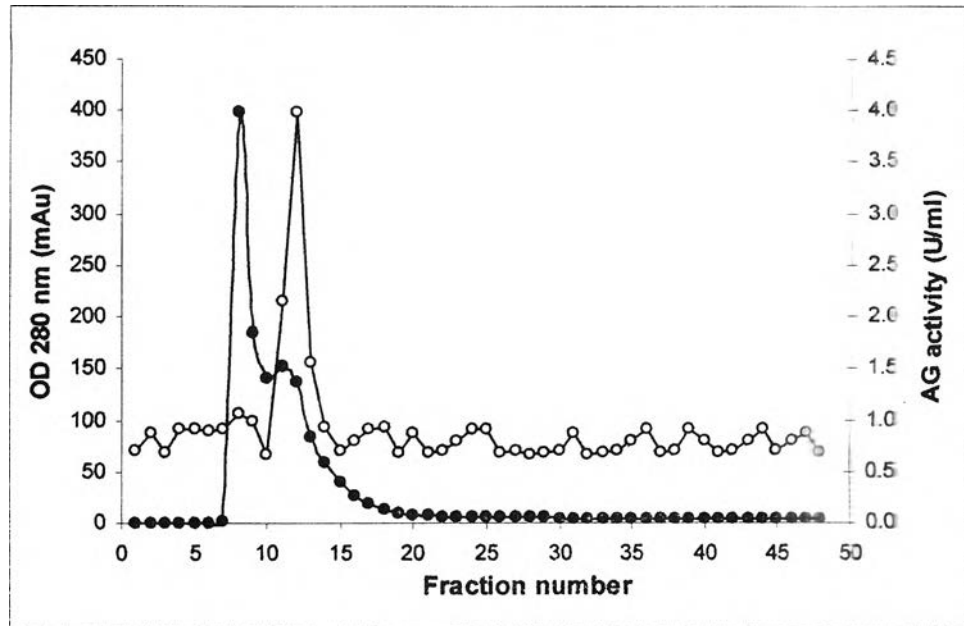
Figure 4.14 AG on a gel filtration (Superdex 200) column. Unbound peak sample of DEAE – cellulose, 18 mg protein; column, 1.6 x 38 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 mM NaCl (pH 6.3); flow rate, 30 ml/ h; fraction size, 10 ml; —●— , OD at 280 nm; —○— , AG activity.

Fraction (from Superdex 200) containing highest AG activity were concentrated and desalted by centrifugal filter (MWCO 10,000 Da). The retentive solution was separated by SDS – PAGE as in Figure 4.15. Bands of Af (55 kDa), Af2 (52 kDa), and Af3 (73 kDa) were excised from the gel for MALDI – TOF MS.
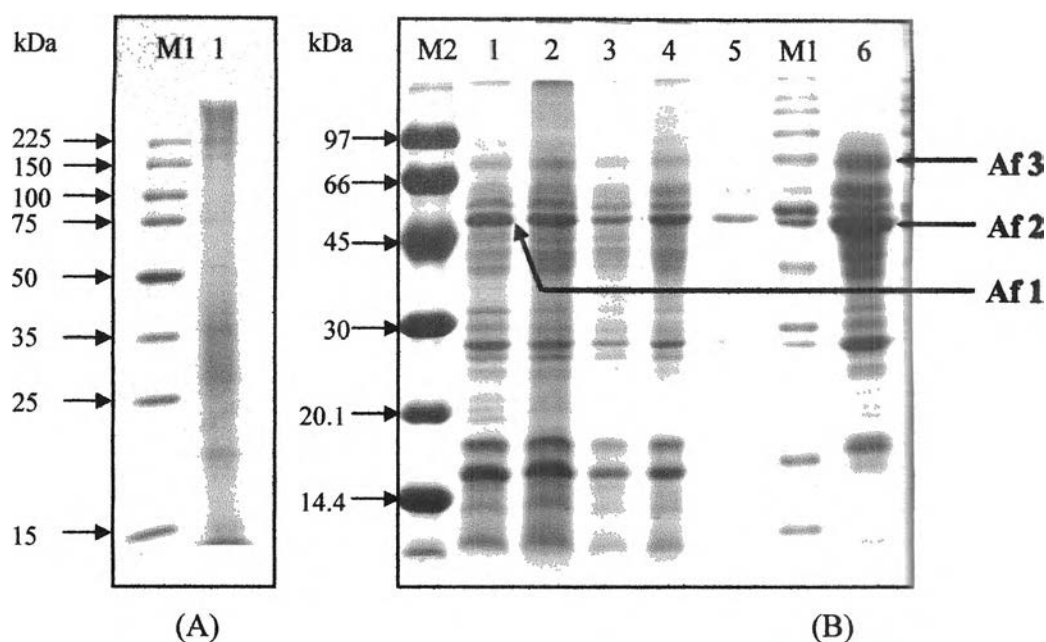
Figure 4.15 CBB staining of SDS - PAGE.

Lane 1 (A): concentrated sample (40 µg) from fraction# 14 of Superdex 200 (from bound DEAE cellulose) (Fig. 4.13)

Lane 1 (B): unbound sample (3 mg) from DEAE cellulose (fraction# 6) (Fig. 4.12)

Lane 2 (B): unbound sample (3 mg) from DEAE cellulose (fraction# 7) (Fig. 4.12)

Lane 3 (B): unbound sample (3 mg) from DEAE cellulose (fraction# 8) (Fig. 4.12)

Lane 4 (B): pooled unbound sample (3 mg) from DEAE cellulose before Superdex 200

Lane 5 (B): highest activity fraction (0.3 mg) from Superdex 200 (fraction# 12) from unbound DEAE cellulose) (Fig. 4.14)

Lane 6 (B): concentrated sample (3.6 mg) from lane 5

Lane M1: broad range protein MW marker

Lane M2: low molecular weight marker

4.5.1.3 Gel filtration (Sephadex G – 150)

The dialyzed unbound peak sample (Fig. 4.12) was chromatographed on a Sephadex G – 150 column equilibrated by 30 mM sodium phosphate buffer containing 100 mM NaCl. Due to Figure 4.16, activity was found in fraction# 40 – 46. The highest fraction contains activity 3.7 u/ ml. Protein in fractions containing activity was separated by SDS – PAGE to determine the purity of purification (Fig. 4.17). Only one band (50 kDa) was visible.
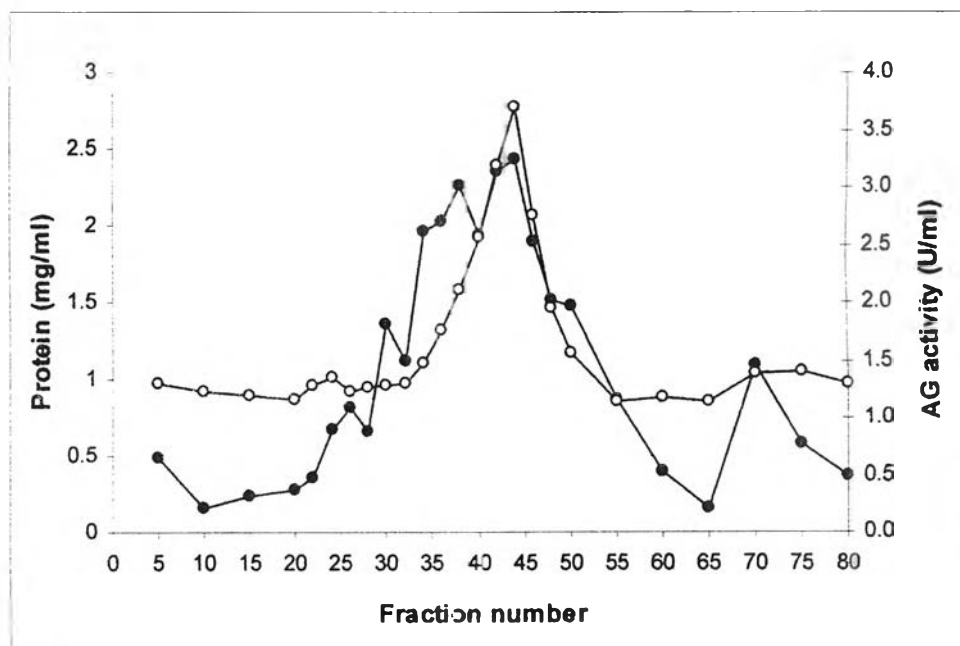
Figure 4.16 AG on a gel filtration (Sephadex G – 150) column. Unbound peak sample of DEAE – cellulose, 10 mg protein; column, 1.5 x 87 cm; equilibrium and elution, 30 mM sodium phosphate buffer containing 100 M NaCl (pH 6.3); flow rate, 15 ml/ h; fraction size, 3 ml; ——●—— , protein concentration (Bradford's assay); ——○—— , AG activity.
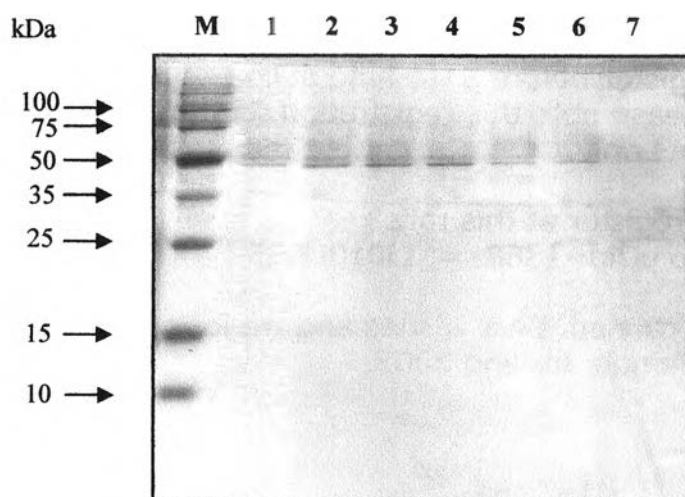
Figure 4.17 SDS – PAGE of high activity fractions from Sephadex G – 150. Lanes 1 – 7 contained fraction# 40 – 46 (100 µg), respectively.

4.5.1.4 Cation exchange (CM – cellulose)

Due to the chromatography on DEAE – cellulose, the protein was not bound on the column. The unbound sample (Fig. 4.13) was dialyzed by 100 mM sodium acetate buffer (pH 5.8) and 20 mM sodium acetate (pH 4.7) overnight, respectively. The dialyzed sample was chromatographed on a CM – cellulose column equilibrated by 20 mM sodium acetate buffer and eluted by 0 – 1 M NaCl. Almost same activity was found in all fractions although protein was eluted from the column (Fig. 4.18). In each fraction, the amount of protein was low (less than 100 mAu by OD 280).
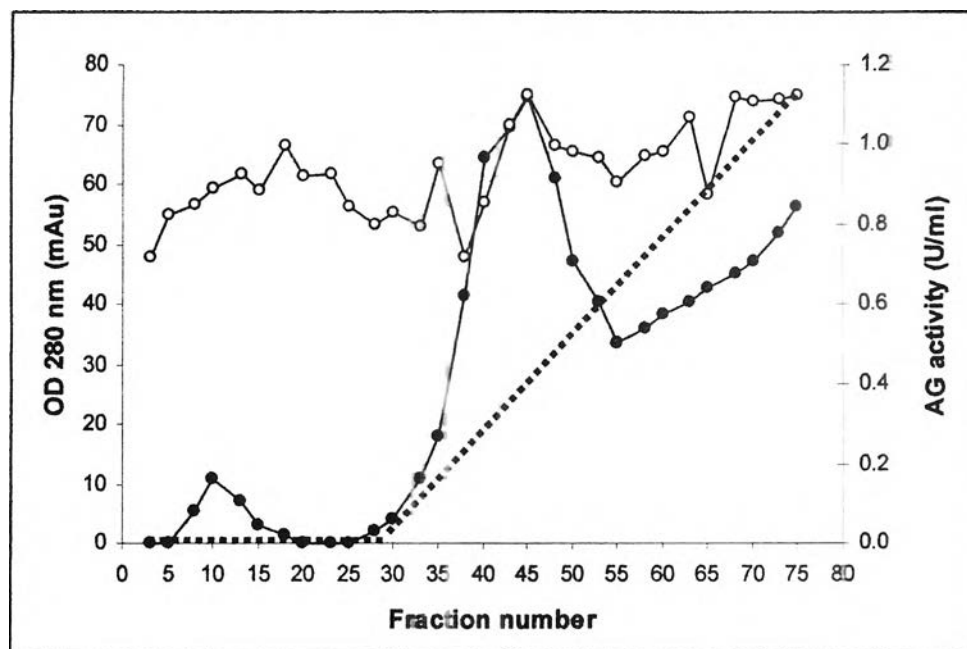
Figure 4.18 AG on CM – cellulose. Unbound peak sample of DEAE – cellulose, 100 mg protein; column, 1.6 x 13 cm; equilibrium, 20 mM sodium acetate buffer (pH 4.7); elution, 0 – 1 M NaCl; flow rate, 60 ml/ h; fraction size, 10 ml;

——●—— OD at 280 nm; —o— , AG activity; ········ , molarity of NaCl

Furthermore, crude protein was injected onto CM – cellulose equilibrated by 20 mM sodium acetate buffer (pH 4.7). The column was developed by a linear gradient of 0 – 1 M NaCl. The AG activity was eluted at second unbound peak (Fig. 4.19). The obtained activity was low (less than 1 u).
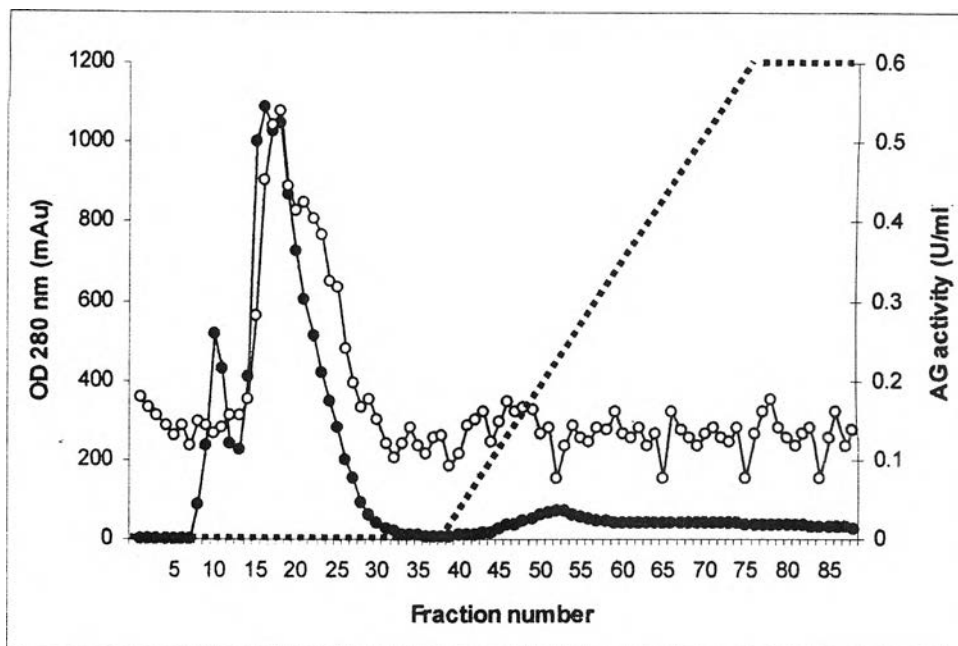
Figure 4.19 AG on CM – cellulose. Crude protein, 300 mg; column, 1.6 x 13 cm; equilibrium, 20 mM sodium acetate buffer (pH 4.7); elution, 0 – 1 M NaCl; flow rate, 60 ml/ h; fraction size, 10 ml; —•— OD at 280 nm; —○— , AG activity; ········· , molarity of NaCl.

4.5.2 Crude protein without ammonium sulfate precipitation

4.5.2.1 Anion exchange (DEAE – cellulose)

In order to avoid the loss of AG activity before chromatography (data from Fig. 4.10), crude protein without being precipitated with ammonium sulfate was applied directly on a DEAE – cellulose under the same condition. The high protein was obtained in unbound (fraction# 2 – 7) and bound (fraction# 28 – 32) peaks. High AG activity was assayed from the unbound peak. SDS – PAGE was shown in lanes 3 – 4 in Figure 4.21.
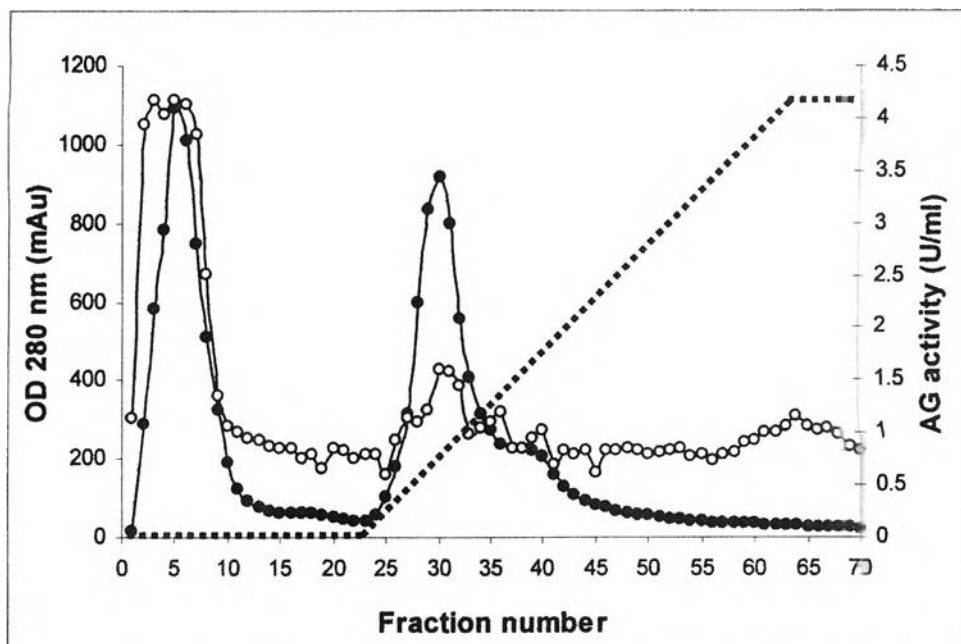
Figure 4.20 Unprecipitated AG on DEAE – cellulose. Crude protein without precipitation with ammonium persulfate, 250 mg; column, 1.6 x 13 cm; equilibrium, 30 mM sodium phosphate buffer (pH 6.3); elution, 0 – 1 M NaCl; flow rate, 60 ml/ h; fraction size, 10 ml; ▬●▬ , OD at 280 nm; ▬○▬ , AG activity; ·········· , molarity of NaCl.

From all procedures of AG purification, it can be summarized in Table 4.3. Specific activity of AS precipitate was lower than crude. Specific activity after DEAE – cellulose was the lowest but it was not greatly different from AS precipitate. AG activity in both bound and unbound peaks from DEAE – cellulose was assayed. The bound peak was separated on Superdex 200 while the unbound peak was separated on Superdex 200, Sephadex G – 150, and CM – cellulose. After second times of purification by gel filtration, specific activity was higher.

Table 4.3 Summary of purification procedures of AG.

Procedure	Total protein (mg)	Total activity (u)	Specific activity (u/ mg)	Yield (%)	Purification fold
Crude	4,065	1,228.5	0.302	100	1
Ammonium sulfate					
(95% saturation)	1,075	195.2	0.182	15.89	0.603
DEAE – cellulose					
- 95% AS	780	133.52	0.171	10.87	0.566
- no AS	20	90	4.5	7.327	14.901
Superdex 200					
- Bound DEAE	44.1	22.8	0.517	1.856	1.712
- Unbound DEAE	52	124	2.385	10.095	7.89
and ultrafiltration	10.46	42.29	4.043	3.443	13.387
Sephadex G – 150					
- Unbound DEAE	60	81.3	1.355	6.619	4.487
CM – cellulose					
- Unbound DEAE	25	38.95	1.558	3.171	5.159
- Crude protein	40.92	26.07	0.637	2.122	2.109

Protein in fractions containing highest AG activity of DEAE – cellulose and Sephadex G – 150 was separated by SDS – PAGE and CBB stain (Fig. 4.21 A). A major band of 100 kDa and minor bands of 35 and 50 kDa were observed in lane 1 but a major band (35 kDa) and a minor band (50 kDa) were observed in lane 3. This may indicate that AS precipitation affects high MW protein.

Protein was denatured and renatured. Many bands of protein were observed on SDS polyacrylamide gel (Fig. 4.21 A) but only one positive band was visible in lanes 2 – 3 of activity gel (Fig. 4.21 B). No AG activity at all in lanes 1 and 4 (Fig. 4.21 B) although lots of protein were detected in Figure 4.21 A.
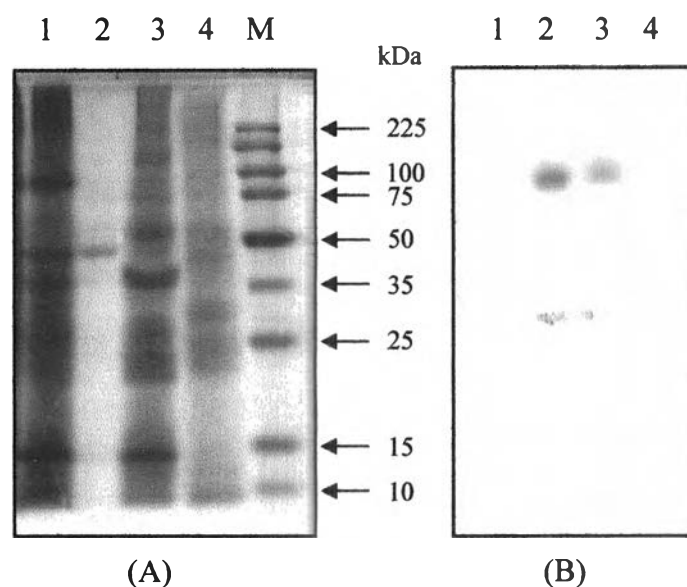
Figure 4.21 CBB staining (A) and activity staining (B) of fractions containing highest activity from DEAE – cellulose and Sephadex G – 150.

Lane 1: unbound fraction with AS (3 mg) on DEAE (Fig. 4.12)

Lane 2: unbound fraction (1 mg) on Sephadex G – 150 (Fig. 4.16)

Lane 3: unbound fraction without AS (1 mg) on DEAE (Fig. 4.20)

Lane 4: bound fraction without AS (1 mg) on DEAE (Fig. 4.20)

Lane M: broad range protein MW marker

Three bands on SDS polyacrylamide gel (Fig. 4.15) were excised. Arrows indicate locations of 3 bands, Af1, Af2, and Af3. Due to the R_f value and log MW of protein marker, MW of Af1, Af2, and Af3 were calculated to be 55, 52, and 73 kDa, respectively (Fig. 4.23). The bands of Af1 and Af2 were selected because they were always found from any chromatography. The Af3 band was excised because its MW (about 73 kDa) was highest MW in SDS – PAGE. The MW of positive band was 93 kDa (Figs. 4.21 and 4.22). Molecular weight of purified AGI, AGII, and AGIII in *A. mellifera* were 98, 76, 68 kDa, respectively (Takewaki *et al.*, 1980 and Nishimoto *et. al.*, 2001). Those bands were later digested by trypsin and analysed for peptide by MALDI – TOF MS at Bioservice unit, Thailand.
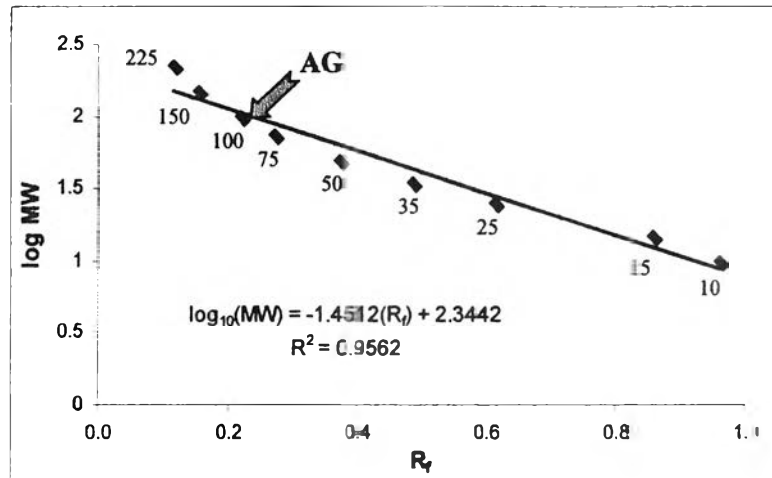
Figure 4.22 Relationship between R_f value and log MW of broad range protein MW marker. MW of AG from Fig. 4.21 was estimated.
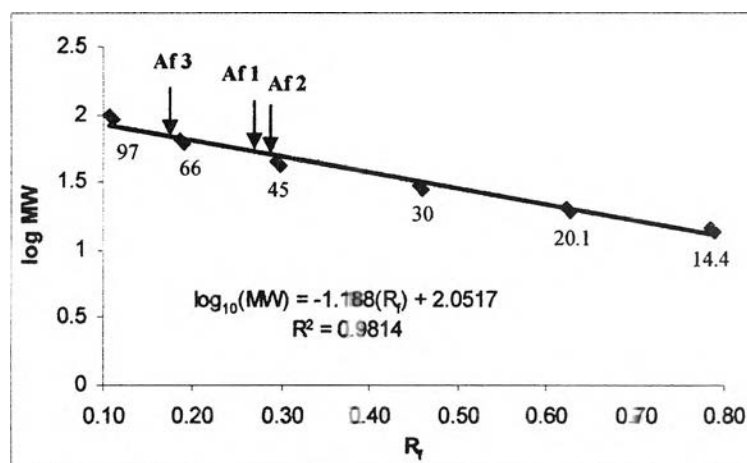
Figure 4.23 Relationship between R_f value and log MW of low MW marker. MW of Af1, Af2, and Af3 from Fig. 4.15 was calculated. The LMW standard marker contains phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and alpha – lactalbumin (14.4 kDa).

4.6 MALDI - TOF peptide mass mapping

Whole body of honeybee was partial purified by 1) various chromatographies; anion exchange (DEAE cellulose), gel permeation (Superdex 200) and 2) by ultrafiltration with centrifugal filter MWCO 10,000 Da. Then, the obtained protein was separated by SDS – PAGE. The protein complement of partial purified AG was sequenced for peptide by matrix assisted laser desorption ionization/time of flight (MALDI – TOF) mass spectrometer.

The peptide mass was searched in SwissProt database in Mascot program (www.matrixscience.com). Peptide matching for sample mass spectra was based on an accuracy of ± 1 Da. The MALDI – TOF mass spectrum of Af3 showed six peptide masses, 1163.543 m/z, 1313.55 m/z, 1719.779 m/z, 1,756.725 m/z, 1977.753 m/z, and 2111.86 m/z, $[M+H]^+$ which were matched to those of AG in $A.$ $mellifera$ (Q17058). The score is 70 which is accepted to be significant (p < 0.05) since it is greater than 67. According to Figure 4.24, the matched peptide is 12% coverage with AG in $A.$ $mellifera$ (based on the M_r of 65.5 kDa).

```
  1 MKAVIVFCLM ALSIVDAAWK PLPENLKEDL IVYQVYPRSF KDSNGDGIGD  50
 51 IEGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS 100
101 DLDNLVSAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP 150
151 GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV 200
201 VLDDMQNVLR FWLRRGFDGF RVDALPYICE DMRFLDEPLS GETNDPNETE 250
251 YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYTDY 300
301 GADFPFNFAF IKNVSRDSNS SDFKKLVDNW MTYMPPSGIP NWVPGNHDQL 350
351 RLVSRFGEEK ARMITTMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG 400
401 CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYKTVNLA 450
451 AEKKDKNSFF NMFKKFASLK KSPYFKEANL NTRMLNDNVF AFSRETEDNG 500
501 SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YNNFNSDIKS ISNNEQVFVS 550
551 ALGFFILISQ DAKFGNF
```

Figure 4.24 The amino acid sequence of AG in $A.$ $mellifera$ (Q17958). Matched peptides are shown in bold letter.

4.7 Comparison of amino acid sequence between deduced amino acid sequence from cDNA and amino acid from MALDI – TOF MS

Comparing amino acid sequence between deduced amino acid sequence from cDNA and from MALDI – TOF MS, most of residues of amino acid are the same except one residue at the position of 32. It is Leucine (L) in deduced amino acid sequence from cDNA but it is Valine (V) from MALDI – TOF MS. However the 2 amino acids have close MW. MW of Valine is 117 Da while Leucine is 131 Da.

```
  1 MKAVIVFCLM ALSIVDAAWK PLPENLKEDL ILYQVYPRSF KDSNGDGIGD 50
                                    EDL IVYQVYPRSF K
 51 IEGIKEKLDH FLEMGVDMFW LSPIYPSPMV DFGYDISNYT DVHPIFGTIS 100
101 DLDNLVSAAH EKGLKIILDF VPNHTSDQHE WFQLSLKNIE PYNNYYIWHP 150
151 GKIVNGKRVP PTNWVGVFGG SAWSWREERQ AYYLHQFAPE QPDLNYYNPV 200
201 VLDDMQNVLR FWLRRGFDGF RVDALPYICE DMRFLDEPLS GETNDPNKTE 250
251 YTLKIYTHDI PETYNVVRKF RDVLDEFPQP KHMLIEAYTN LSMTMKYYDY 300
        IYTHDI PETYNVVR                              YYDY
301 GADFPFNFAF IKNVSRDSNS SDFKKLVDNW MTYMPPSGIP NWVPGNHDQL 350
    GADFPFNFAF IK
351 RLVSRFGEEK ARMITTMSLL LPGVAVNYYG DEIGMSDTYI SWEDTQDPQG 400
401 CGAGKENYQT MSRDPARTPF QWDDSVSAGF SSSSNTWLRV NENYKTVNLA 450
451 AEKKDKNSFF NMFKKFASLK KSPYFKEANL NTRMLNDNVF AFSRETEDNG 500
        NSFF NMFKK              EANL NTRMLNDNVF AFSR
501 SLYAILNFSN EEQIVDLKAF NNVPKKLNMF YNNFNSDIKS ISNNEQVKVS 550
551 ALGFFILISQ DAKFGNF
```

Figure 4.25 Comparison of amino acid sequences between deduced amino acid sequence from cDNA (upper line) and amino acid sequence from MALDI – TOF MS (lower line). The different amino acid was showed by underline letter.

4.8 Two – dimensional electrophoresis

Crude protein precipitated by 95% ammonium sulfate was desalted and separated on 2 – D electrophoresis. Most MW of protein was low in the range of 14.4 – 45 kDa as in Figure 4.26. The distinguished spots were detected in range of pH 3 – 8.5. Owing to result of MALDI – TOF, MW of AG is about 73 kDa. Affirmatively, the assumed AG protein is marked in circle with the expected MW of about 73 kDa at pH 5.5.
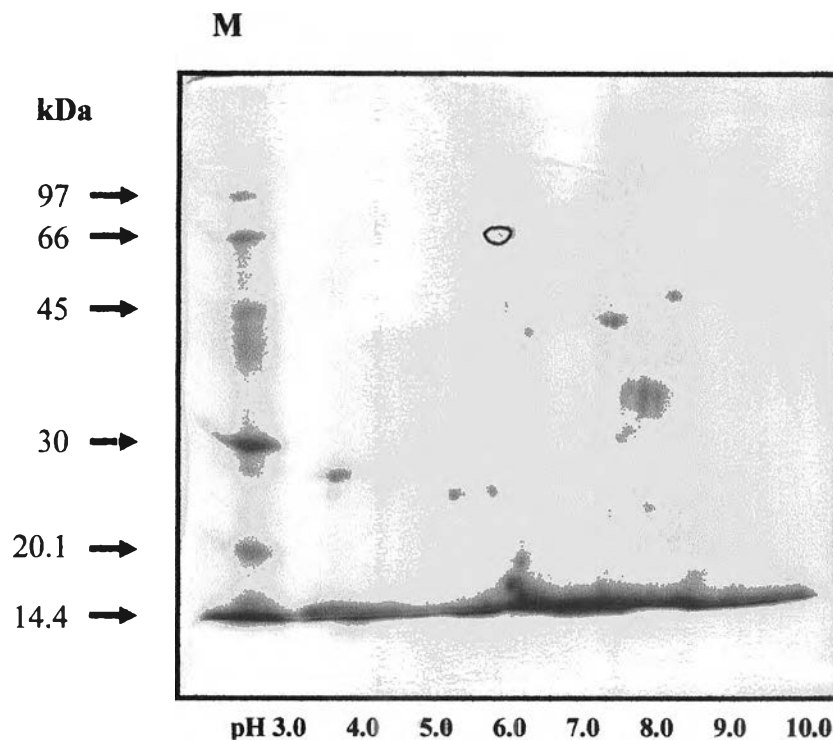
Figure 4.26. Two - D electrophoresis of crude protein (2 mg). Lane M contained low MW marker.

4.9 Optimum conditions for AG

A fraction containing activity peak from Superdex 200 (Fig. 4.14) was selected to study optimum conditions for AG activity. The optimum parameters (pH, temperature, selective concentration of substrate, and incubation time) were measured as mentioned in Materials and Methods. Three replications were performed. The average value was calculated and used to plot a graph.

The obtained optimum pH of partial purified AG was 5 (Fig. 4.27). The optimum temperature was 55°C (Fig. 4.28). The selective concentration of substrate was 80 mM (Fig. 4.29) and the optimum incubation time was 40 min (Fig. 4.30).

4.9.1 Optimum pH
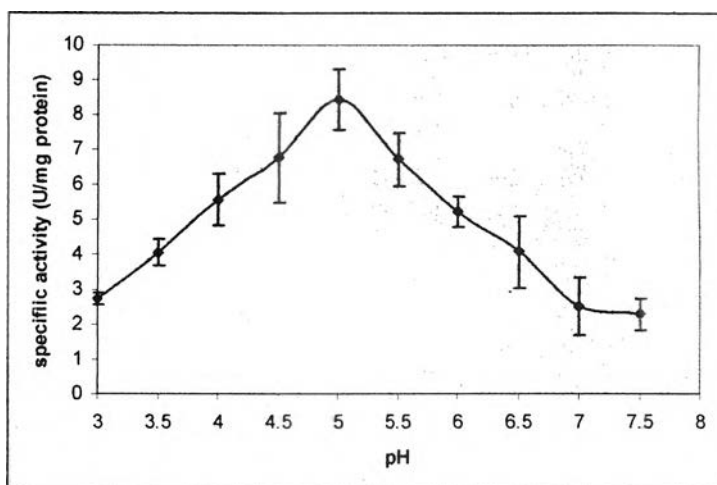
Figure 4.27 The optimum pH of partial purified AG in *A. florea*. Briton – Robinson buffer at various pHs ranging between 3.0 – 7.5 was used. The optimum pH was 5.0.
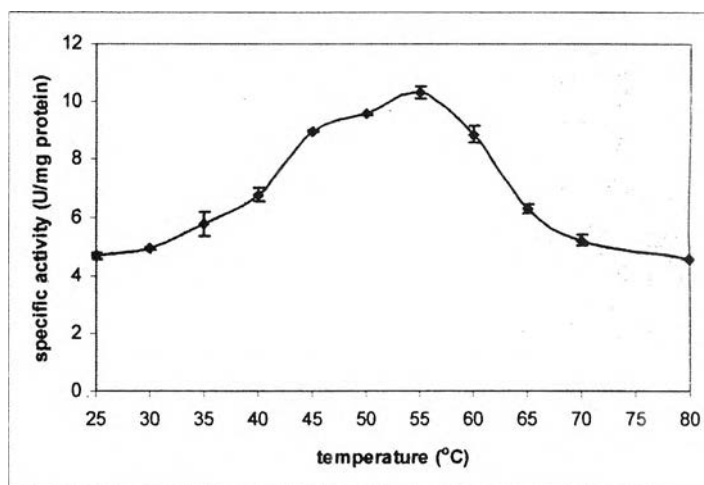
4.9.2 Optimum temperature

Figure 4.28 The optimum temperature of partial purified AG of *A. florea*. The reaction mixture in acetate buffer (pH 5.0) containing 0.1 M sucrose was incubated at various temperatures ranging among 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, and 80°C for 10 min. The optimum temperature was 55°C.

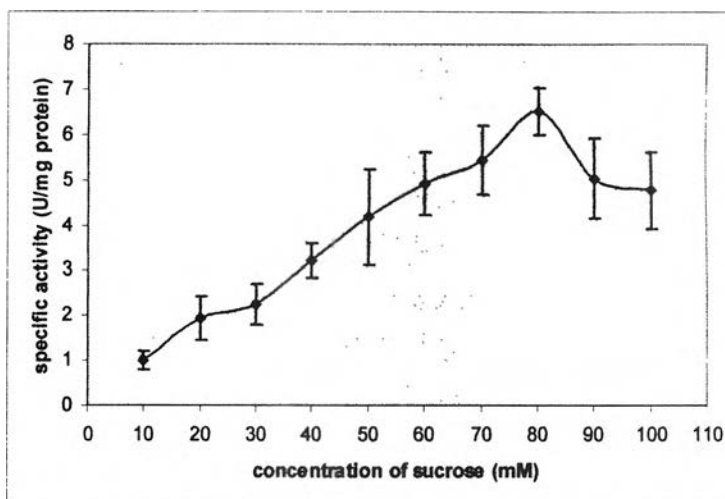### 4.9.3 Selective concentration of substrate for partial purified AG

Figure 4.29 The optimun sucrose concentration of partial purified AG in *4. florea*. The reaction mixture was incubated with sucrose at various concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM, respectively. The optimum concentration of sucrose was 80 mM.

4.9.4 Optimum incubation time of partial purified AG
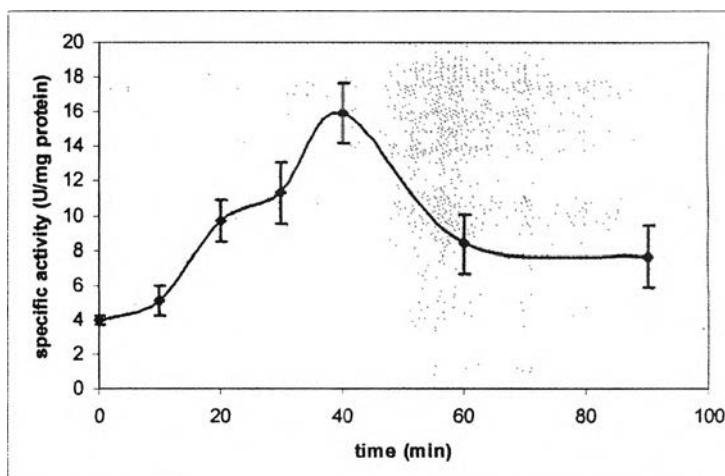

Figure 4.30 The optimun incubation time of partial purified AG in *A. florea*. The reaction mixture was incubated at 55°C for 10, 20, 30, 40, 60, and 90 min, respectively. The optimum incubation time was 40 min.