Transcriptional Activation of Cu/Zn Superoxide Dismutase And Catalase Genes by Panaxadiol Ginsenosides Extracted From *Panax ginseng*

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ABSTRACT

Superoxide dismutase (SOD) and catalase constitute the first coordinated unit of defense against reactive oxygen species. Here, we examined the effect of ginseng saponins on the induction of SOD and catalase gene expression. To explore this possibility, the upstream regulatory promoter region of Cu/Zn superoxide dismutase *(SOD1)* and catalase genes were linked to the chloramphenicol acetyltransferase *(CAT)* structural gene and introduced into human hepatoma HepG2 cells. Total saponin and panaxatriol did not activate the transcription of *SOD1* and catalase genes but panaxadiol increased the transcription of these genes about 2-3 fold. Among the panaxadiol ginsenosides, the Rb_2 subfraction appeared to be a major inducer of *SOD1* and catalase genes. Using the deletion analyses and mobility shift assays, we showed that the *SOD1* gene was greatly activated by ginsenoside Rb_2 through transcription factor AP2 binding sites and its induction. We also examined the effect of the content ratio of panaxadiol extracted from various compartment of ginseng on the transcription of *SOD1* gene. Saponin extract that contains 2.6-fold more PD than PT from the fine root increased the *SOD1* induction about 3-fold. These results suggest that the panaxadiol fraction and its ginsenosides could induce the antioxidant enzymes which are important for maintaining cell viability by lowering level of oxygen radical generated from intracellular metabolism.

Introduction

All aerobic organisms produce reactive oxygen species, such as superoxide radicals and hydrogen peroxide, both spontaneously and as a result of enzymatic activity of oxidative enzymes (Fridovich, 1983 ; Malmstrom, 1982). Cu/Zn superoxide dismutase *(SOD1)* is a protective enzyme responsible for maintaining a low level of superoxide radicals within the cell by converting superoxide radicals to oxygen and hydrogen peroxide, which is in turn changed to oxygen and water by catalase. Catalase is found in virtually all aerobic cells and is partly responsible for protecting cells against the toxic effects of hydrogen peroxide. Overproduction of *SOD1* alone or catalase alone had only minor incremental effects on the average life span of *Drosophila* (Orr and Sohal, 1994). It is therefore of importance to explore the inducer which triggers the expression of antioxidant enzymes.

Panax ginseng C. A. Mayer (Araliaceae) is one of the most popular natural tonics and has been shown to possess various biological activities such as a protein anabolic effect, antitumor activities and an inhibitory effect of tumor angiogenesis and metastasis (Sato *et al.*, 1994). Moreover, ginseng has the advantage that it is free from harmful side effects. Saponin, the active fraction of ginseng, is classified largely into two groups, panaxadiol(PD) and panaxatriol(PT) saponins, which differ in sugar moiety at the position of carbon-3,-6 and -20 (Sanada *et al.*, 1974; Shoji, 1974).

Previously, we have reported that the transcription of *SOD1* was regulated by many transcription factors (Kim *et al.*, 1993 ; Kim *et al.*, 1994; Seo *et al.*, 1996; Kim *et al.*, 1996; Seo *et al.*, 1997; Kim *et al.*, 1997, Yoo *et al.*, 1998; Chang *et al.*, 1998). Here, we examined the total saponin, panaxadiol, panaxatriol and each major ginsenosides for the induction of *SOD1* and catalase genes. Panaxadiol appeared to be a good inducer for both genes and its ginsenoside Rb_2 was the best one. Also, we demonstrated that the activation of *SOD1* gene by ginsenoside $Rb_2$was mediated by the transcription factor AP2. In addition, we tested the effect of the content ratio of panaxadiol extracted from various parts of ginseng root on the transcription of *SOD1* gene.

Materials and Methods

Total saponin, panaxadiol, panaxatriol and ginsenoside-Rb_1, -Rb_2 -Rc and -Rd were isolated from six years old red ginseng by thin layer chromatography and high performance liquid chromatography (Sanada *et al.*, 1974; Shoji, 1974) and supplied by Korea Ginseng and Tobacco Research Institute. Plasmid SODCAT, which contains the promoter region of *SOD1* gene, was constructed as follows: the 1.7-kilobase pair *Bam*HI/*Sma*I fragment (nucleotides -1633 to +85) of the promoter region from the rat *SOD1* gene (Kim *et al.*, 1993) was inserted into pBLCAT2 (Luckow and Schutz, 1987). Plasmid pBluCLCAT, which contains the promoter region of the catalase gene, was prepared by polymerase chain reaction using synthetic oligonucleotide primers 5'-CGCGGATCCATAATACT-TACATTAGCGTATGGCA-3' 5'(-AAACTGCAGAACACTGCACTGCACTGCAGGAGGCCTCG-GCT-3', which amplified the genomic sequence from -554 to -27 of the promoter. The cloned fragment was inserted into the *Bam*H1 and *Pst*1 sites of the multiple cloning site of pBluCAT, which was created by inserting the CAT gene of pBluscriptSK. The sequence of the cloned catalase upstream region was verified by DNA sequence analysis. For the construction of pAP2w, the oligonucleotide of AP2 binding sequence, which was corresponding to SV40 AP2 binding site, cloned into the *Bam*HI site of pBLCAT2. Two copies of the AP2 consensus oligonucleotide were introduced. The plasmid pRSP-305AP2m was constructed as follows; DNA fragment (*Hae*II-*Nhe*I) from pRSP-305 was excised and ligated with synthetic oligonucleotide containing the mutated AP2 binding site. The AP2 binding sequences located at -134 and -118 were replaced with mutated sequence (at -134, from CCCCGCCC to CCATATCC; at -118, from CCCCGCGG to CCATATGG).

Human HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM)/ 10% fetal calf serum/ penicillin G sodium at 100 units per ml/ streptomycin sulfate at 100 μg per ml/ amphotericin B at 250 ng per ml. Cells were seeded into 60 mm plastic culture dishes (30-50% confluence) for 24 hours prior to transfection. An equal amount (3.0 pmole) of SODCAT or pBluCLCAT construct was transfected to the cells by the calcium phosphate DNA coprecipitation method (Chen and Okayama, 1988). A 5μg sample of pRSV β-gal plasmid (Edlund *et al.*, 1985) was also introduced in all experiments to correct for variations of transfection efficiency. Ginseng saponins were added to culture medium at 36 hours after transfection and the cells were maintained for an additional 22 hours. To determine the maximum induction time of Rb_2, the growth medium was removed and the Rb_2 was added to the cells at 50μM (SODCAT) or 100μM (pBluCLCAT) in phosphate-buffered saline (PBS). After treatment for 30 min at 37℃, the growth medium was added back to the cells and incubation was continued for proper intervals as indicated. Cell extracts were first assayed for β galactosidase activity (Sambrook *et al.*, 1989) and equal quantities of proteins were assayed for CAT activity on the basis of β-galactosidase activity. The CAT assay was performed as described (Gorman *et al.*, 1982).

Results and Discussion

In this study, we demonstrated the effect of ginseng saponin on the expression of *SOD1* and catalase genes. The promoter region-CAT fusion plasmids of *SOD1* and catalase genes SODCAT and the pBluCLCAT, respectively, were introduced into HepG2 cells. The TS and PT treatments had no effect on both *SOD1* and catalase gene expression, but PD induced the transcription of both *SOD1* and catalase genes about 2-3 fold, respectively (Fig.1 A,B).

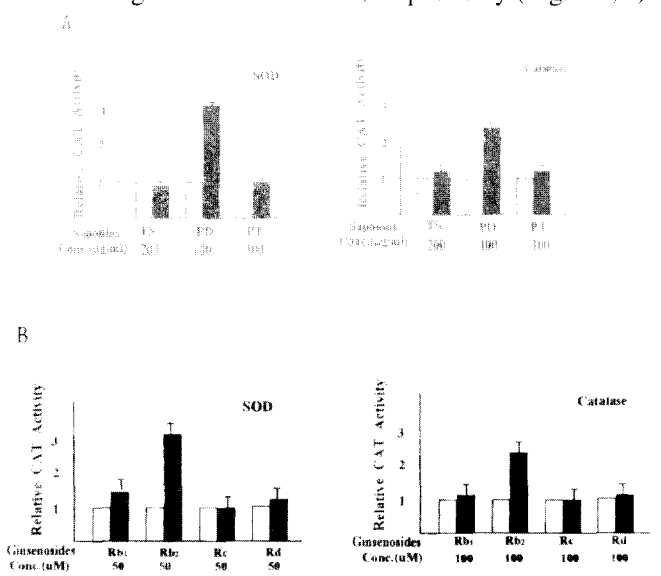

Fig.1.
(A) Effect of ginseng saponins on the induction of *SOD1* and catalase genes. Total saponin (TS), panaxadiol(PD) and panaxatriol(PT) were treated as indicated. CAT activity was measured 70 hours after reporter plasmid transfection. Solid bar indicates the CAT value with saponins and open bar without saponins. The results of CAT assay are representatives of the mean ± S.D. of three independent experiments.
(B)Effect of major ginsenosides of PD on the induction of *SOD1* and catalase genes.
After transfection for 48 hr , ginsenoside - Rb1 ,- Rb2, - Rc and - Rd were added for 22 hr before CAT assay. Solid bar indicates the CAT value with saponins and open bar without saponins. The results of CAT assay are representatives of the mean ± S.D. of three independent experiments.

PD consisted of ginsenoside-Rb_1,-Rb_2, -Rc, -Rd and some other minor fractions. Therefore, we tested which subfraction of PD had an effect on the induction of the *SOD*1 gene. Fig.1C and D show that ginsenoside Rc and ginsenoside Rd had no effect on either induction or inhibition, whereas Rb_2 was 2-3 fold more potent activator than Rb1 on both *SOD1* and catalase expression. By increasing concentrations of Rb_2 up to 100μM for *SOD1* and 200 μM for catalase genes, the induction was gradually increased (Fig. 2A). To characterize the induction profile for the time course incubation, the medium of transfected cells was removed and treated with 50 μM Rb_2 in PBS for 30 min. After the incubation, the cells were washed with PBS and then fresh growth medium was added. The induction profile of *SOD1* reached a peak at 2h after Rb_2 addition and then declined gradually (Fig. 2B). Catalase reached a peak after a relatively short period incubation time with Rb_2 and maintained the induction level up to 4h (Fig. 2B). These results confirmed panaxadiol ginsenoside Rb_2 as a potent inducer of both enzymes.

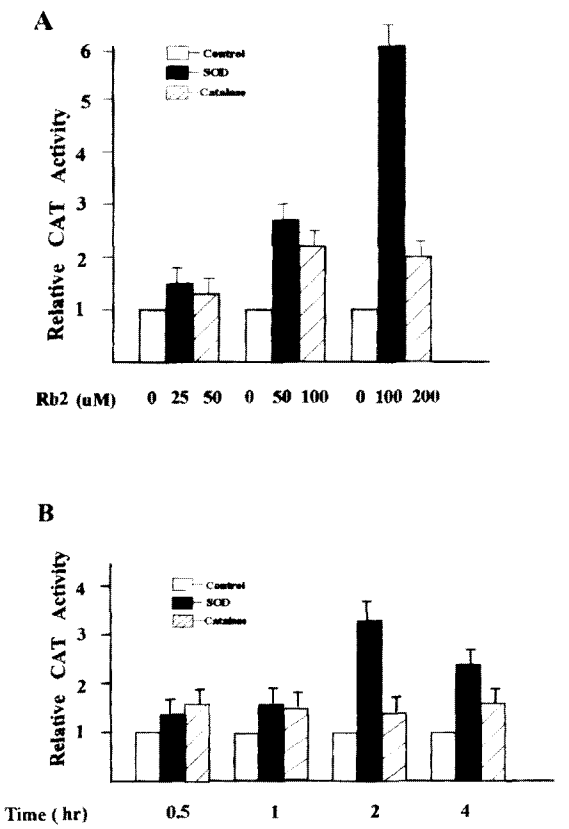

Fig.2.
Effects of the concentration and treatment time of Rb_2 on expression of the *SOD1* and catalase genes.
(A) Treatment of the cell with increasing amounts of Rb2.
(B) Time course induction profiles. To determine the maximum induction time for Rb_2, the growth medium was removed and Rb2 was added to the cells at 50 μM(SODCAT) or 100μM (pBluCLCAT) in phosphate-buffered saline (PBS). Treatments were maintained for 30 min at 37℃, and the Rb_2 was removed and new growth medium was added for the continuing incubation as indicated. The results of CAT assay are representatives of the mean ± S.D. of three independent experiments.

To identify the target sequence of Rb_2 in the upstream region of the *SOD1* gene, deletion mutants were prepared and transfected into the HepG2 cell with or without Rb_2 in the medium. In Fig. 3B, Rb_2-specific induction was observed in every construct except the one which had minimal promoter (pRSP-55). Rb_2 induced *SOD1* expression about 3-fold, regardless of its promoter strength, under the appropriate conditions. These results showed that the target sequence for Rb_2-specific induction was located in the -305 to -55 region. This region was the proximal part of the *SOD1* gene promoter that consisted of a large number of transcription factor binding site. As shown in schematic view of *SOD1* promoter in Fig. 3A, it is possible that induction of *SOD1* gene by Rb_2 is mediated by activation of specific transcription factors. We demonstrated that AP2 factor was increased by Rb_2 treatment and its binding site was in the promoter region of the *SOD1* gene. Rb_2 did not affect the level of CAT activity of the AP2 mutant plasmid, pRSP-305AP2m, whereas the CAT activity of the wild type pRSP-305 increased about 5-fold in the natural context (Fig. 3D). Consistent with these results, almost identical results were also obtained from the heterologous systems (Fig. 3C). Transcriptional activation by AP2 involves the 52-kDa AP2 protein binding to a specific DNA motif found in the *cis*-regulatory region of the gene (Williams and Tjian, 1991). AP2 has also a crucial role in the

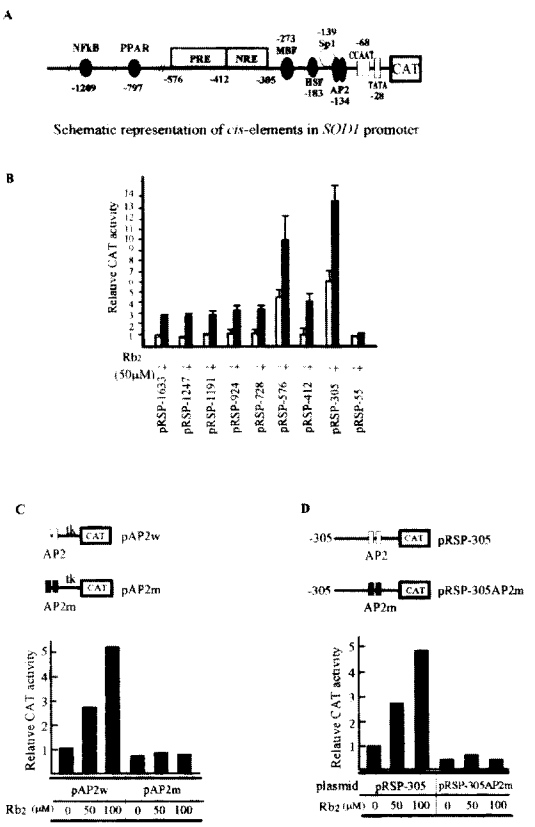

Fig. 3.

Fig. (A) Schematic representation of the putative transcription factor binding sites and (B) deletion analysis of the upstream region of *SOD1* gene for the identification of target region responsible for the induction by the ginsenoside Rb_2. The numbers in the name of plasmid represented the deletion end-point. CAT activities with (+) or without (-) Rb_2 were indicated by solid bar and blank bar, respectively. (C) Transcription activation through the AP2 binding site by ginsenoside Rb_2. Schematic diagram of synthetic AP2 (pAP2w) and mutated AP2 oligonucleotide (pAP2m) in the heterologous promoter (tk) attached to CAT. Effect of Rb_2 on the synthetic AP2 and mutated AP2 in the heterologous promoter was shown with histogram. (D) Schematic diagram of the *SOD* promoter from nt -305 to +85 (pRSP-305) and mutated AP2 sites of the promoter (pRSP-305AP2m) attached to CAT. Effect of Rb2 on AP_2 and mutated AP_2 in the natural context was shown with histogram. Note that mutations in the synthetic AP_2 sites and in the natural context abolished the induction activity by Rb_2.

induction of the antioxidant enzyme heme oxygenase 1 by heme (Lavrovsky *et al.*, 1994). These results showed us a novel action mechanism of ginseng saponin on *SOD*1 transcription, and could also provide a molecular link between ginseng saponin intake and its inhibitory effects on aging and mutation by radical oxygen (Kim *et al.*, 1996).

To investigate the possibility of ginseng for the functional food as an inducer of antioxidant enzymes, we tested the effect of the content ratio of panaxadiol to panaxatriol in the extract from various parts of ginseng root on the transcription of *SOD1* gene. We observed that the transcriptional activation of *SOD1* was proportioanl to the contents ratio of panaxadiol ginsenosides. Consistent with these results, the extract prepared from the real-fine root, which contains the higher ratios of panadiol to panaxatriol about 2.6 fold, increased the *SOD1* transcription about 3-fold(Fig. 4). These results suggest that the extract from the fine root, PD, and Rb_2 would serve as inducers of antioxidant enzymes. Considering difficulties of purification and limited quantity, our results suggest that the saponin extract from the fine root and the panaxadiol fraction, which are relatively easier to obtain than its subfractions, would be useful candidate for the induction of antioxidant enzymes.

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References

Chang, M. S., Yoo, H. Y. and Rho, H. M. (1998). Submitted for publication.

Chen, C., and Okayama, H. (1987). High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7**:2745-2752.

Edlund, T., Walker, M. D., Barr, P. J. and Rutter, W. J. (1985). Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5' flanking elements. *Sci* **230**: 912-916.

Fridovich, I. (1983). Superoxide radical : an endogeneous toxicant. *Annu Rev Pharmacol Toxicol* **23**: 239-257.

Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2**:1044-1051.

Kim, Y. H., Yoo, H. Y., Jung, G. H., Kim, J. Y. and Rho, H. M. (1993). Isolation and analysis of rat genomic sequence encoding Cu/Zn superoxide dismutase. *Gene* **133** : 267-271.32.

Kim, H. T., Kim, Y. H., Nam, J. W., Lee, H. J., Rho, H. M. and Jung, G. (1994) Study of 5'-flanking region of human Cu/Zn superoxide dismutase. *Biochem Biophys Res Commun* **201**, 1526-1533.

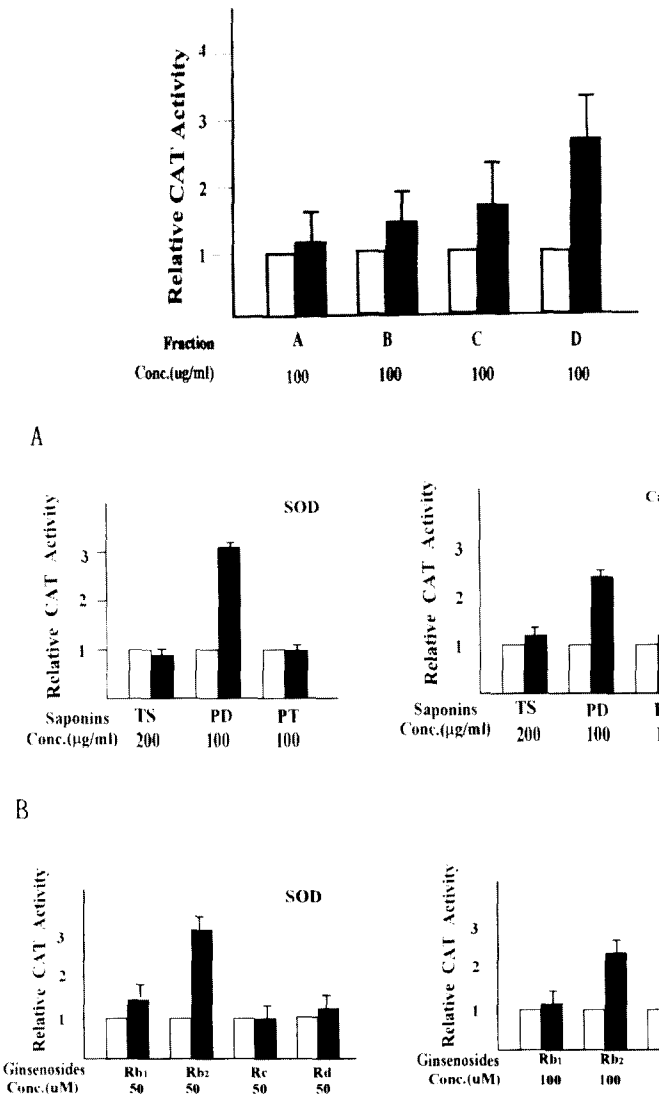

Fig.4.
Effects of saponins extracted from different parts of ginseng root, which contains different ratio of PD to PT, on the induction of *SOD1* gene. Ginseng soponins were added to culture medium at 36h after transfection and the cells were maintained for additional 22h. CAT activities with or without saponins were indicated by the solid bars and blank bars, respectively. Fraction A, main root ; B, lateral root ; C, fine root; D, real-fine root.

Kim, Y. H., Park, K. H., and Rho, H. M. (1996). Transcriptional activation of the Cu,Zn-Superoxide Dismutase Gene through the AP2 site by Ginsenoside Rb_2 Extracted from a medicinal Plant, *Panax ginseng* . *J Biol Chem* **271**: 24539-24543.7.

Kim, Y. H., Yoo, H. Y., Chang, M. S., Jung, G. and Rho, H. M. (1997). C/EBPα is a major activator for the transcription of rat Cu/Zn superoxide dismutase gene in liver cell. *FEBS Lett* **401**, 267-270.

Lavrovsky, Y., Schwartzman, M. L., Levere, R. O., Kappas, A., and Abraham, N. G. (1994). Identification of binding sites for transcription factors NF-kB and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci U.S.A.* **91** :5987-5991

Luckow, B., and Schutz, G. (1987). CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res* **15**:5490.

Malmstrom , B. G. (1982). Enzymology of oxygen. *Annu Rev Biochem* **51**: 21-59.

Orr, W. C. and Sohal, R. S. (1994). Extention of Life-Span by overexpression of Superoxide Dismutase and Catalase in Drosophila melanogaster. *Sci* **263**: 1128-1130.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanada, S., Kondo, N., Shoji, J., Tanaka, O. and Shibata, S. (1974). Studies on the saponins of ginseng. I. Structures ooff ginsenoside -Ro, -Rb_1, -Rb_2, -Rc and -Rd. *Biol Pharm Bull* **22**: 421-428.

Sato, K., Mochizuki, M., Saiki, I., Yoo, Y. C., Samukawa, K. and Azuma, I. (1994). Inhibition of Tumor Angiogenesis and Metastasis by a Saponin *Panax ginseng,* Ginsenosid-Rb_2. *Biol Pharm Bull* **17**: 635-639.

Seo, S. J., Kim, H. T., Cho, G., Rho, H. M. and Jung G. (1996). Sp1 and C/EBP-related factor regulate the transcription of human Cu/Zn SOD gene. *Gene* **178**, 177-1858.

Seo, S. J., Kang, S. S., Cho, G., Rho, H. M. and Jung, G. H. (1997) C/EBPα and C/EBP β play similar roles in the transcription of the human Cu/Zn SOD gene. *Gene* **203**, 11-15.

Shoji, S. (1974). Some Chemical Studies on Ginseng. In : Proceedings of International ginseng symposium, pp69-76. The Central Research Institute, Office of Monopoly, Republic of Korea.

Williams T., and Tjian R. (1991) Analysis of the DNA-binding and actiavation properties of the human transcription factor AP-2. *Genes Dev.* **5** : 670-682

Yoo, H. Y., Chang, M. S. and Rho, H. M. (1998) Submitted for publication.