CHAPTER II
LITERATURE REVIEWS

Glucocerebrosidase (GBA) is an enzyme that catalizes the breakdown of the glycolipid glucosylceramide to glucose and ceramide (Futerman *et al.*, 2004). Glucocerebrosidase gene is responsible for acid-β-glucosidase (Winfield *et al.*, 1997). It is located on chromosome 1q21. Mutations in this gene result in glucosylceramide accumulation mainly in macrophage and some other tissues of the reticuloendothelial system (Charrow *et al.*, 2004). These accumulations induce symptoms belonging to Gaucher disease, an inherited metabolic disorder among persons of Ashkenazi Jewish descent.

Gaucher disease is devided into 3 phenotypes which are based on the absence or presense and severity of the primary central nervous system (CNS) involvement; the type 1, non-neuronopathic; the type 2, acute neuronophathic and the type 3, subacute neuronopathic (Grabowski *et al.*, 1996). This classification system has implications for the clinical pathologic and molecular aspects of the disease.

Gaucher disease type 1 lacked neuronopathic involvement and was the most common variant. The estimation of the disease incident was in between 1/60,000 and 1/360,000 in the Caucasian population. Onset of clinical manifestations or age at diagnosis could occur from childhood to adulthood with a median age of 16 years. Affected children had been described with massive hepatosplenomegaly, an abnormal increase in the sizes of liver and spleen, as well as liver function abnormalities, pancytopenias and extensive skeletal abnormalities.

Gaucher disease type 2 was the most severe variant and was characterized by progressive involvement of the CNS, particularly the brainstem. This variant was rare (< 1/500,000 live births). Early and late onset forms differed in the age of onset and death which occurred by 2-3 month. The distinction between type2 and type3 was not absolute, but type2 had more aggressive, rapidly progressing brainstem discase with death occurs within 24-36 months.

Gaucher disease type 3 had less severe and more variable CNS involvement than type 2. Reliable estimates of the disease frequency were not available, but it probably

occurred in < 1/100,000 live births. These variants might not be completely clinically distinct and might not distinguished molecularly even with full allele characterization.

Clinically therapeutic approach to Gaucher disease was divided into 4 main treatments; 1) biophosphonate supplement, 2) bone marrow transplantation, 3) gene transfer ring and 4) enzyme replacement therapy (Grabowski *et al.*, 1996).

Although biophosphonate compound was used in clinical treatment, this compound inhibited pathological bone resorption by mechanisms that was not well understood (Grabowski *et al.*, 1996). They appeared to inhibit osteoclast activity and possibly recruitment of new osteoclasts. Bone density, as measured by quantitative computerized tomography demonstrated increases in overall bone density.

Bone marrow transplantation, as well as gene transfer, was developed successfully but in rare cases. In one well documented case, enzyme reconstitution to expect levels was achieved in visceral organs but no increase in brain enzyme levels was found (Grabowshi *et al.*, 1996). In these situations, enzyme replacement therapy should be the best alternative of treatment.

Enzyme replacement therapy (ERT) is currently available treatment for Gaucher disease. In the past, Gaucher patients were treated with Ceredase®, a natural glucocerbrosidase extracted from placenta. Although this treatment had been developed, it still had side-effects. The patients who were treated with Ceredase® developed IgG antibody against it. Recent development of recombinant form of glucocerebrosidase (figure 2.1) had been addressed under trade name Cerezyme®. Comparing with natural glucocerebrosidase, there was less incidence in those who were treated with Cerezyme® (Grabowski *et al.*, 1996).

Cerezyme® was the recombinant glucocerebrosidase, manufactured by Genzyme Corporation. Several researches on Cerezyme provide evidences of this application the most efficient treatment for ERT among 3000 patients worldwide (Futerman *et al.*, 2004). The treatment also significantly improved many clinical symptoms such breakdown of stored glucosyl ceremide, reduction in liver and spleen size and bone pain decrease (Charrow *et al.*, 2004).

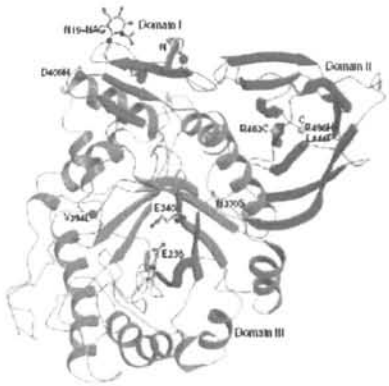

Figure 2.1 Glucocerebrosidase enzyme (Dvir *et al.*, 2003)

However, Cerezyme® had several disadvantages. First, it required life-long, intravenous infusions at least every 2 weeks (Futerman *et al.*, 2004). Second, it was one of the most expensive drugs in the world, making its application unsuitable for patients who could not afford. The latter was probably because of its production procedures.

Most of recombinant protein production system at present are based on bacteria, yeasts and mammalian cells. Although prokaryote production system can be manipulated in large-scale fermentation, and can grow rapidly and express high-level of recombinant proteins, several posttranslational modifications, including signal peptide cleavage, protein folding, disulfide bond formation and glycosylation, might not be carried out in prokaryotes. As a result, complex proteins that were produced in prokaryotes are not always properly folded or processed to provide the desired degree of biological activity. Simple therapeutic proteins, such as insulin, interferon or human growth hormone, did not require folding or extensive posttranslational processing to be biologically active (Gomord and Faye, 2004).

In the case of Cerezyme®, which was carried out in mammalian cell host (chinese hamster ovary), its production processes involved in expensive in fermentation with high in operating costs but provided only low yields with difficult to scale up to large volume and had possibility in contamination by viruses and prions (Gomord and Faye, 2004).

The growing in clinical demand for complex recombinant proteins had created substantial interest in developing new expression system for the production. In the past decade, plants had emerged as a suitable alternative to the current production systems for recombinant proteins. The capacity of plants for low-cost production of high-quality, much

safer and biologically active mammalian proteins is well documented (Gomord and Faye, 2004).

Plants were one of many possible systems for producing proteins. Potentially plants provided a cheap source of recombinant proteins, such as industrial enzymes, technical materials and biopharmaceuticals. For example, Giddings (2001) had estimated that the production costs of recombinant proteins in plants could be between 10 and 50 times lower than those for producing the same protein in *E. coli*.

Apart from economic advantages, there were qualitative benefits favoring the use of transgenic plants as factories for producing recombinant proteins, particular in pharmaceutical proteins. Expression systems in animal cells synthesize mammalian recombinant-products correctly, but are expensive and sensitive to environmental changes, especially when cultured on an industrial scale. Whereas the production of protein from transgenic plants was higher in biomass production, relatively fast gene to protein time, low operating costs, capability of eukaryotic post translational modifications, low risk of human pathogens, lacking of endotoxins, but high protein yields (Giddings, 2001).

Unicellular green microalgae were attracted interest for biological research as a simple model functionally as for higher plants, and from a biotechnological perspective, as a natural source of high value compounds for use in health and medical applications. Fundamental researches had focused on several algae. *Chlamydomanas reinhardtii* was developed as a plant system for the study of photosynthetic and other metabolic pathways, the biogenesis of subcellular components and the cell-cycle control. Other species such as *Haematococcus*, *Chlorella* and *Euglena* carotenoid produced such as β-carotein, astaxanthin and canthaxanthin used as pigments in food products and cosmetics, vitamin A antioxidant supplements, health food products and feed additives for poultry, livestock, fish and crustaceans (Walker, 2005). Among them *Dunaliella* was major system for β-carotein production. Microalgae could potentially be a future commercial source of a number of other vitamins including vitamin C, E and B_{12}. Pharmacologically active compounds had also been reported in microalgae, including compounds with anticancer, antimicrobial, antiviral and various neurological activities as well as lipids that were rich in long chain polyunsaturated

acids of interest in the treatment of heart disease and as anti-inflammatory agents (Lee *et al.* 1985).

The harvesting of products from microalgal culture had a number of advantages from an industrial perspective, which had led to an increased interest in the biotechnology of this group of organisms. Micro algae are photoautotrophic obviating the need for an exogenous carbon source for energy and making their large-scale culture comparatively cheap. Several microalgae grew in saline to hyper-saline waters and thus their large-scale culture did not compete with conventional agriculture for the limited resources of arable land and fresh water. In addition, microalgae such as *Dunaliella* sp., *Haematococcus* sp. and *Chlorella* sp. which did not produce toxins and were classified as safe as food sources (Walker, 2005). Naturally, the potential for large-scale culture were unsuitable for conventional crops, making microalgae a desirable target for both increased production of natural high-value compounds and biological factories for the synthesis of recombinant proteins.

While the utility of *Chlamydomonas* sp. as a model organism had been facilitated by the development of genetic transformation procedures (Stevens and Purton, 1997), the lack of efficient genetic transformation systems for other microalgal species had been a limitation in the manipulation of other organisms (Walker *et al.*, 2005).

The basis of traditional methods used to transform microalgae is to cause temporal permeabilization of the cell membrane, enabling DNA molecules to enter the cell while preserving viability.

One method of permeabilization involved the use of glassbeads and polyethylene glycol (PEG) (Purton, 1998). This method had been successfully used for the gene transfer of cell-wall-deficient mutants or wild-type cells of *Chlamydomonas* sp. following enzymatic degradation of the cell wall, and is routinely used owing to its simplicity and efficiency. It was interesting to investigate whether this method could be applied to other species of microalgae.

Similarly, a method using silicon carbide (SiC) whiskers was described for *Chlamydomonas* sp. without removal of the cell wall (Dunahay, 1993). This silicon carbide-mediated transformation had previously been used for the genetic manipulation in several higher plants and some microalgae species such as *Amphinidium* sp. and *Symbiodinium*

(Lohris and Miller, 1998). However, because SiC whisker was difficult to purchase and could be a health harzard, this method was not recommendable at first choice (Leon-Banarez *et al.*, 2004).

The introduction of genes by electroporation had been carried out in many types of cells and organisms. Stable transformants of both wall-less and walled strains of *Chlamydomonas* sp. (Shimogawara *et al.*, 1998) and *Dunaliella* sp. (De-Gui *et al.*, 2004) had been obtained in this way with high efficiency.

Another method used for the transformation of microalgae was the particle bombardment. This method used DNA-coated particles of gold or tungsten that were accelerated into the target cells by a helium-driven gun. It is routinely used for the transformation of plant cells and tissues and also for transformation of prokaryotes and eukaryotic organelles. This method had been successful in the transformation of diatoms (Apt *et al.*, 1996; Falciatore *et al.*, 1999 and Zaslavskaia *et al.*, 2000).

Eukaryptic algae are phylogenetically heterogenous. Stable nuclear transformations had been reported in only 3 euharyotic microalgal groups: chlorophytes, diatoms and dinoflagellates. Recently, chloroplast transformation of the unicellular red algae *Porphyridium* sp. had been reported (Lapidot, 2002).

Diatoms belong to the division Chrysophyta and were of biotechnological interest because they had silica-based cell walls and an be used to obtain pharmaceutical products, or as food source in aquaculture. Five different species had been transformed: *Phaeodactylum tricornutim* (Apt *et al.*, 1996), *Cyclotella cryptica* , *Navicular saprophila* (Dunahay *et al.*, 1996), *Cylindrotheca fusiformis* (Fischer, 1999) and *Thalassiosira weissflogii* (Falciatore *et al.*, 1999).

Dinoflagellates are a group of unicellular eukaryotic alveolar algae that constitute an important part of marine phytoplankton. *Amphinidium* sp. and *Symbiodinium* sp. are the 2 species of this group that had been successfully transformed (Lohuis and Miller, 1998).

The fresh water algae *Chlamydomonas* sp. was the first and best-studied transformation system of the chlorophytes or green algae group. With complete sequencing of its genome, these well documentated researches had made *Chlamydomonas* an excellent model system for diverse areas of applications. However, other chlorophytes with important

economical value, such as *Dunaliella* sp. had not been well observed since its lack in report on efficient genetic transformation system.

Dunaliella sp. (figure 2.3) was considered an interesting model for gene expression study because it had properties closer to plant protoplast than other algae. Moreover, over 80% of the world's supply of natural β-carotein came from this halophilic green alga. The taxonomy of D. salina was as followed.

Division Chlorophyta

Class Chlorophyceae

Oder Dunaliellales

Family Dunaliellaceae

Dunaliella salina (Ben-Amotz and Avron, 1982)

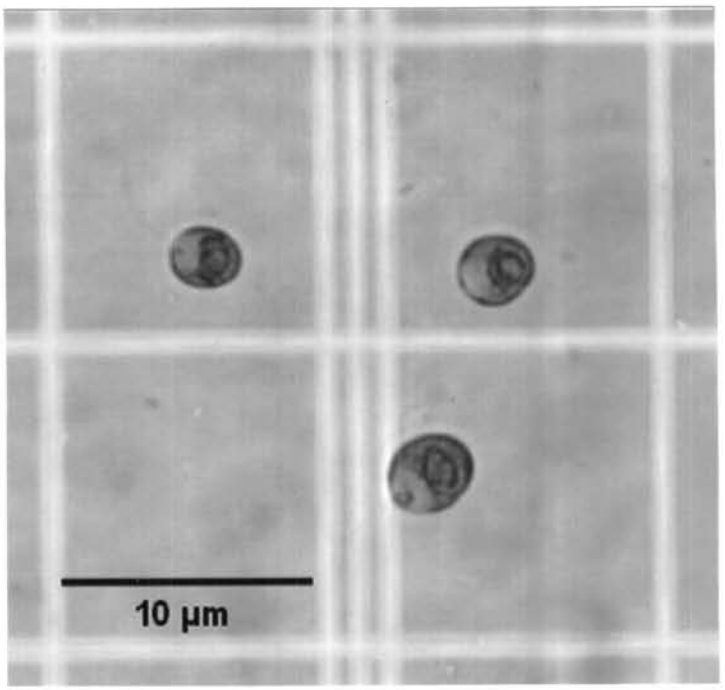

Figure 2.3 *D. salina* cells in 40X objective lens

D. salina, the unicellular eukaryotic green microalgae, was an ideal organism for the study of salt resistance mechanisms because it could grow in low-salt media to saturated salt solutions (Ben-Amotz *et al.*, 1982). Because of the lack of rigid polysaccharide cell wall, it was a natural protoplast. Foreign gene could be easily transformed into the cells and recombinant proteins could also be easily purified to meet the demand of safety and

efficiency (De-Gui *et al.*, 2004). In addition, it could be rapidly cultured in an inexpensive medium containing simple salts making its cost of protein-production very low. These advantages made *D. salina* a good bioreactor for large-scale production.

Although genetic transformation in *Dunaliella* sp. was not widely observed, it might be an alternative model for the researches in genetic transformation. Walker *et al.* (2005) used the *RbcS* promoter and 3' untranslated region of *D. tertiolecta* combining with *ble* gene for transformation in *Chlamydomonas reinhardtii*. The result were shown to drive expression of the the bleomycin resistance gene.

In 2004, De-Gui *et al.* observed the stable expression of hepatitis B surface antigen gene in *D. salina*. The DNA was introduce by electroporation and HBsAg expression were maintained stable for at least 60 generations.

These advantages had provide *D. salina* an interesting organisms to study a transformation system, protein expression and use it as a protein production system.