EnzymeWave

Volume 2

New Biological Methods Applied to the Koji Fermentation System
A Novel Enzyme Preparation for D-Amino Acid Production
A Novel Protein Modifying Enzyme : Protein-Glutaminase
The Current Status of "Green Chemistry" in Japan

AMANO

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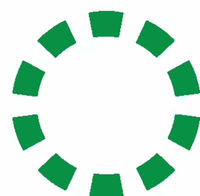
From the Editor,

The world exposition, EXPO 2005 AICHI, is scheduled to open in Aichi Prefecture, Japan on March 25, 2005 and run through September 25, 2005. The theme of EXPO 2005 AICHI is "Nature's Wisdom". Nature, the source of all life on earth, has bestowed wisdom on mankind enabling us to develop the technologies that made our modern industrial world possible. However, this wisdom was not given to mankind to wreak irreparable damage to the environment. The purpose of EXPO 2005 AICHI then is to provide a pause in our frantic pace to develop new technology and reflect once more on "nature's wisdom" to find a more harmonious relationship between technology and our environment. Technology is not inevitably harmful to our environment and the future of mankind is very much dependent on finding this new balance.

Throughout history Japan has been very conscious of nature as expressed in Japanese art and culture. However, because of the lack of land and natural resources and very rapid industrialization, Japan has experienced a rapid deterioration of the environment. Amano Enzyme Inc. believes that as a supplier of specialty enzymes we can play a part in re-establishing a balance between technology and nature. Amano believes that enzymes, which are nature's catalyst, express "nature's wisdom" and offer an environmentally friendly alternative to many industrial processes. Amano, with its head office in Aichi Prefecture, proudly sponsors EXPO 2005 AICHI.



Rendition of the Global Loop

New Biological Methods Applied to the Koji Fermentation System

Katsuya Gomi,
Professor, Graduate School of Agricultural Science, Tohoku University

Traditional Koji fermentation

There is a long history of utilizing solid fermentation to produce fermented foods in Asia. In particular, a solid fermentation process called "koji- making" has been applied for production of various traditional foods in Japan. Koji-making is the process of growing selected fungi (for example *Aspergillus oryzae*, *Aspergillus sojae* and *Aspergillus awamori*) on steamed grain; the resulting koji is used as a source of enzymes necessary for the production of foods.

In Japan, solid fermentation has not only been used for food production, but also for the production of industrial enzymes. In 1894, a famous scientist, Johkichi Takamine, developed an industrial production process for the enzyme called "Takadiastase" (amylase) based on solid fermentation of *Aspergillus oryzae* grown on steamed wheat bran. This was the first commercial scale production of a microbial enzyme and the same process is still used today.

Solid fermentation "koji-making" is an efficient process for enzyme production, and the resulting enzyme preparations are useful for food processing. However, the koji-making process is problematic because culture conditions are difficult to maintain and scale up potential is limited. Therefore much effort has been spent developing liquid fermentation processes for production of koji-like enzyme preparations. However, all attempts have failed because some enzymes necessary for koji could not be produced using liquid fermentation conditions. This suggests that there are some unique mechanisms for enzyme production by solid fermentation. Recently, advanced research based on molecular biology has shed light on these unique mechanisms.

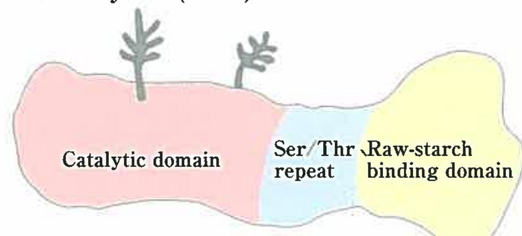
Discovery of a specific glucoamylase gene expressed during solid fermentation.

Glucoamylase is the most important enzyme used in sake brewing. The glucoamylase gene was cloned and modified using genetic engineering techniques in order to increase glucoamylase production. The glucoamylase protein was first purified from rice-koji and a partial amino acid sequence was determined. Oligonucleotide probes were then synthesized based on the amino acid sequence, and the glucoamylase gene (*glaA*) was cloned from a cDNA library of *Aspergillus oryzae*. The cDNA fragment containing *glaA* was subcloned into an expression vector and *Saccharomyces cerevisiae* was transformed and utilized for the expression of *glaA*. Unexpectedly, the resulting gene product had a raw-starch degrading activity which is not present in glucoamylase isolated from rice-koji. When *Aspergillus oryzae* was transformed with multiple copies of the *glaA* gene an increase in glucoamylase activity produced by liquid fermentation was observed, but no increase in glucoamylase activity was found when the enzyme was produced by solid fermentation.

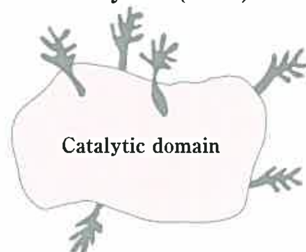
These findings lead us to suspect that there must be another gene for glucoamylase and its expression is specific for solid fermentation. To demonstrate this hypothesis, we cloned another glucoamylase gene (*glaB*) using a cDNA library prepared from *Aspergillus oryzae* cultivated on solid medium. The *glaA* and *glaB* genes are different in DNA sequence. Glucoamylase coded by *glaA* has a catalytic domain in the N-terminal region, a raw-starch binding domain in the C-terminal region and a short serine/threonine rich domain in the central region. In contrast, glucoamylase coded by *glaB* has a catalytic domain in the N-terminal region which is 55% homologous with the N-terminal region of *glaA*, but has neither a short serine/threonine rich domain nor a raw-starch binding domain.

Johkichi Takamine
(1854-1922)

Glucoamylase (GlaA)



Glucoamylase (GlaB)



The *glaB* gene is strongly expressed by solid fermentation, but not by liquid fermentation. On the other hand, *glaA* is expressed at nearly the same level in both liquid fermentation and solid fermentation. However, the expression level of *glaB* is much greater than *glaA* when both are produced by solid fermentation. Glucoamylase encoded by *glaB* is the predominant glucoamylase protein in rice-koji. This is the first report of an enzyme produced in solid fermentation but not in liquid fermentation.

Further research has revealed unique features of *glaB* expression. Some stress factors (high temperature and low moisture) which cause poor growth of hyphae can stimulate the expression of *glaB*. Such stress conditions are similar to koji-making conditions established during the long history of sake brewing. In the koji-making process, rice grain is steamed to harden its surface. The surface hardness provides stress that inhibits growth of hyphae. The temperature is kept high (40°C) during the late stage of the koji process and this high temperature promotes removal of moisture from steamed rice grain. These conditions induce production of the *glaB* encoding glucoamylase, which is the most important enzyme for sake brewing. This is one example of a modern scientific explanation for an old traditional development process.

Table 1 : Expression of glucoamylase and acid protease encoding genes in ***Aspergillus oryzae***

Gene	Expression	
	Liquid	Solid
glaA	+	+
glaB	–	++
pepA	–	+++

Other solid fermentation specific genes.

There appears to be many other genes expressed specifically during solid fermentation. These genes could be important genes controlling the growth, metabolism and anabolism pathways of fungi. For example, the acid protease from *Aspergillus oryzae* and the acid-tolerant amylase from *Aspergillus kawachii* are only slightly produced during liquid fermentation but are produced to a very significant level by solid fermentation. Research into the expression of the *Aspergillus oryzae* acid protease gene (*pepA*) demonstrated that *pepA* was expressed at very low levels in liquid fermentation and expressed at high levels in solid fermentation. These results suggest that *PepA*, like the *glaB* encoded glucoamylase is produced only during solid fermentation. However, unlike the *glaB* gene, the expression of the *pepA* gene is repressed at high temperature (40°C). In koji-making, acid protease is produced at the early stage of the process (25°C), but is not produced at the late stage of the process (40°C). This early stage specific production of acid protease seems to be controlled at the level of transcription by temperature.

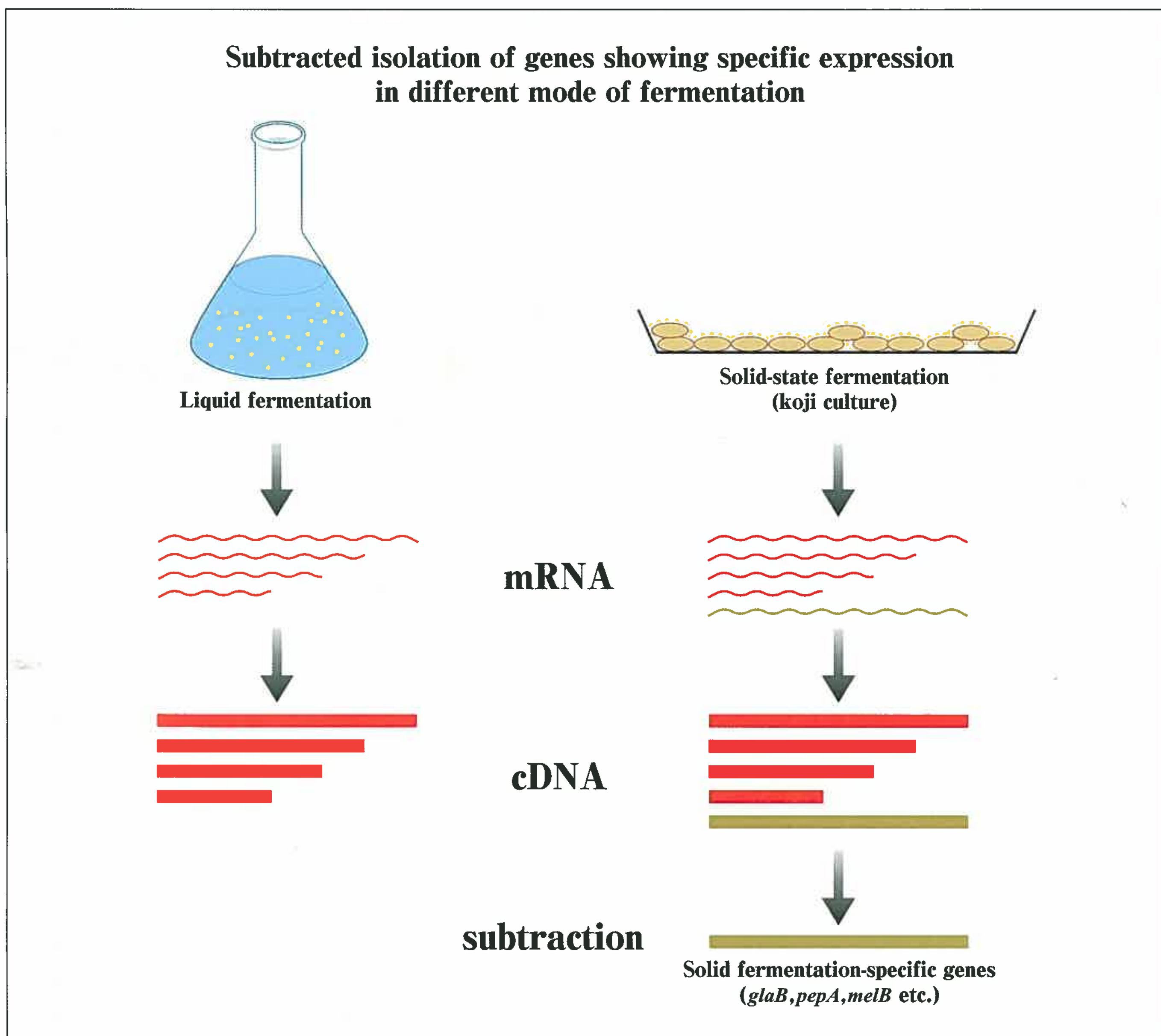
Another example of an enzyme gene expressed differently during solid and liquid fermentation is the tyrosinase gene of *Aspergillus oryzae*. Two distinct tyrosinase genes, *melO* and *melB*, were cloned; *melO* was expressed specifically by liquid fermentation while *melB* was expressed specifically by solid fermentation.

Future research strategy

Recently a project for *Aspergillus oryzae* genome analysis has been initiated in Japan. In a few years, the DNA sequence of all genes in *Aspergillus oryzae* will be available. Prior to the *Aspergillus oryzae* genome project, a project to collect information based on partial nucleotide sequences of expressed genes (Expressed Sequence Tags) in *Aspergillus oryzae* was started. Currently EST information for about 6,000 genes has been collected including information from genes expressed in solid fermentation using wheat bran (raw material for industrial solid fermentation) and genes expressed in liquid fermentation. In parallel with the EST project, differences in gene expression during liquid fermentation and solid fermentation were identified by using subtractive gene technology.

The results from these studies have identified solid fermentation specific genes. At present, the only gene specific for solid fermentation with a known function is the *glaB* gene. Therefore there must be unknown genes which might have important function for fungal growth in solid fermentation.

In the near future DNA microarray technology will provide analysis of expression levels for a vast number of genes. This in turn should elucidate expression networks for gene regulation and allow for a more rational design of expression systems for the production of industrially important enzymes more efficiently by solid fermentation. Conversely, this knowledge may also enable the production of solid fermentation specific enzymes by liquid fermentation.



A Novel Enzyme Preparation for D-Amino Acid Production

D-amino acids are very important chiral intermediates utilized in the development of various pharmaceutical compounds including antibacterial agents and anti-diabetic agents.

D-amino acids are currently produced by a chemical synthesis process because production of D-amino acids by fermentation is known to be very difficult. However since chemical synthesis of amino acids results in a chiral mixture of D- and L- amino acids, several methods have been developed for the resolution of DL-amino acids. As an enzymatic method, L-aminoacylase has been used for selection and extraction of D-amino acids from a racemic mixture at a commercial scale (Figure 1). However, there are some problems associated with this process: low yield of the final product, complexity of the process and poor cost performance.

The use of D-aminoacylase in a direct selection process to separate D-amino acids from a racemic mixture of amino acids is described in Figure 2. Although the advantages of this process have been known for years, the unavailability of a commercial source for the enzyme has prevented its use on a large scale.

Dr. Moriguchi at Oita University in Japan has described the isolation of D-aminoacylase from the bacterium *Alcaligenes sp.* (*Protein Expression and Purification*,7,395(1996)). Amano Enzyme Inc. has cloned the gene using a recombinant *E. coli* system and has successfully developed D-aminoacylase as a commercial product. D-aminoacylase "Amano" efficiently hydrolyzes N-Ac-D-amino acids, especially N-Ac-D-methionine, N-Ac-D-phenylalanine and N-Ac-D-leucine to D-amino acids and acetic acids.
D-aminoacylase "Amano" should prove beneficial for the commercial production of D-amino acids.
(Patent No. WO00/78926)

Figure 1 : Production of D-Amino acid with L-Aminoacylase

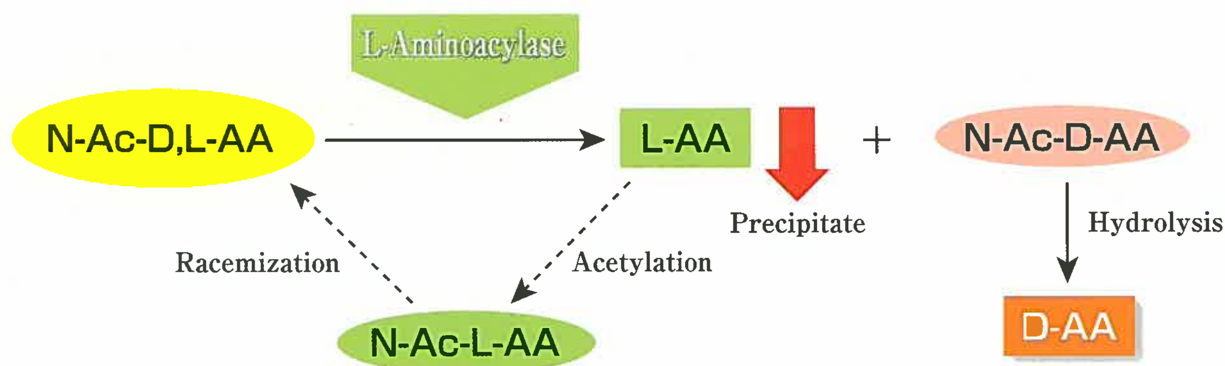
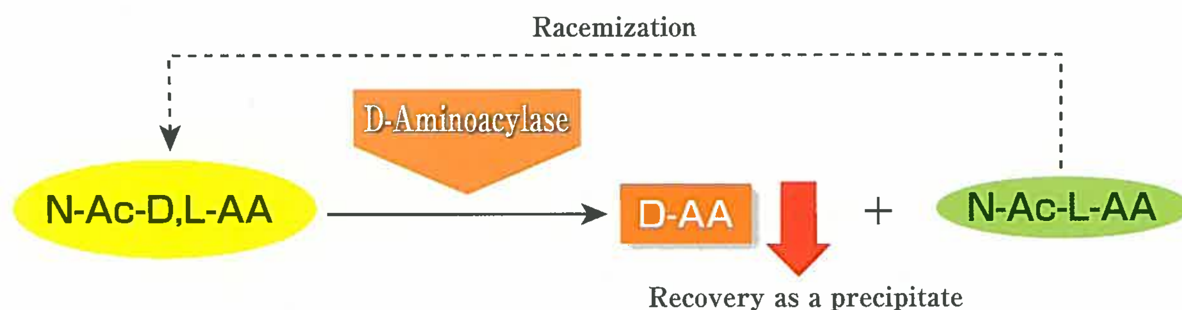


Figure 2 : Production of D-Amino acid with D-Aminoacylase



A Novel Protein Modifying Enzyme : Protein-Glutaminase

Protein is an important ingredient in food systems. In addition to the nutritional benefit, protein contributes to the textural and sensory properties of food. For instance, egg white protein is an excellent foaming agent in bakery products and the gelling property of milk protein is utilized for cheese and yogurt making. These properties are referred to as "protein functionality" (Table). An improvement in the functionality of under utilized plant proteins such as wheat and corn gluten would expand their usefulness to the food industry and because these proteins are widely available in large quantity any increase in food applications would have a positive impact on feeding the world's population.

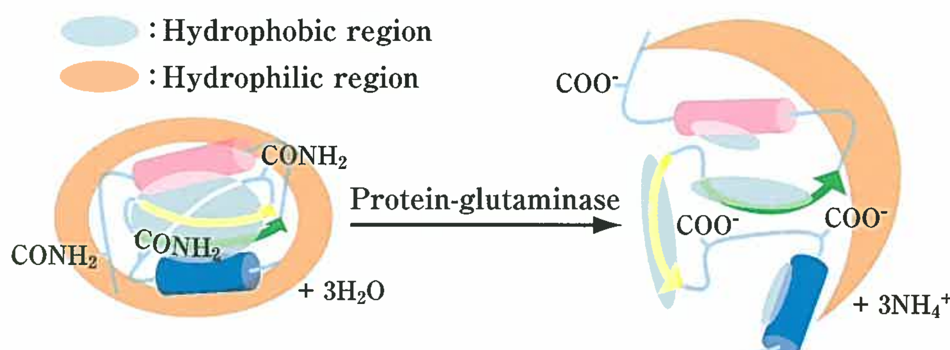
The enzymatic modification of proteins is a promising method for improving protein functionality because it is highly specific and compared to the chemical modification process is environmentally friendly. Limited proteolysis, utilizing proteases from animal, plant and microbial sources, has been widely used for this purpose. The use of chymosin in cheese making, one of the oldest and best-known examples of enzyme utilization in human history, can be classified in this category. Recently a microbial transglutaminase (protein cross-linking enzyme), first discovered by the R&D group at Amano Enzyme Inc. and now being commercialized with the collaboration of Ajinomoto Co., Inc., has had a significant impact on the food protein industry.

Amano has continued to search for new types of protein modification enzymes. An extensive screening effort for potential enzymes has resulted in the discovery of a novel protein-deamidating enzyme (*Appl. Environ. Microbiol.*, ***66***, 3337 (2000)). The enzyme converts the glutaminyl residues in protein to glutamyl residues and is called protein-glutaminase (*Eur. J. Biochem.*, ***268***, 1410 (2001)). More than 95% of the glutaminyl residues of some food proteins, such as milk casein and wheat gluten, are deamidated by this enzyme. The isoelectric point of deamidated protein is lowered as a result of the increase in negatively-charged carboxyl groups; the product is a protein with good solubility at the more acidic pH of many food systems. Protein deamidation also causes an alteration of the protein folding structure due to the newly formed negative charges. This unfolding leads to exposure of hydrophobic regions, resulting in a protein with improved amphiphilic character that would make deamidated protein an ideal emulsifier or foaming agent. In addition to improvement in protein functionality, protein-glutaminase can be used for many applications in the food industry, including dough softening in the baking industry, enhancement of umami taste (glutamic acid) in hydrolyzed vegetable protein, and increase protein digestibility (Patent No. EP0976829A2). This novel enzyme will be commercialized by Amano in the near future.

Table. Protein Functionality in Food Systems

Functionality	Protein type	Food
Solubility	Whey proteins	Beverages
Viscosity	Gelatin	Soups, gravies, salad dressings, desserts
Water binding	Muscle and egg proteins	Meat sausages, cakes, breads
Gelation	Muscle, egg and milk proteins	Meats, gels, cakes, bakeries, cheese
Emulsification	Muscle, egg and milk proteins	Sausages, bolongna, soup, cakes, dressings
Foaming	Egg and milk proteins	Whipped toppings, ice cream, cakes, desserts

Improvement of protein functionality



The Current Status of "Green Chemistry" in Japan

Green Chemistry is the use of chemical technology to reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products.

"Strategic Forum for Green Biotechnology" was organized in 1999 to promote green chemistry in Japan. In 2000, the forum proposed a plan for implementing the principles of green chemistry in the Japanese chemical industry. The following goals for 2010 were outlined in the plan:

- **Introduction of biotechnology into the chemical industry.**
  The chemical industry accounts for 13% of energy consumption in Japan. It was estimated that if biotechnology were utilized in 30% of current chemical processes, 1% of total energy consumption in Japan would be saved. The market for products in this sector resulting from the use of biotechnology was estimated to be $70 billion.

- **Utilize biomass as an alternative energy source to petroleum.**
  A reduction of 4 million kilo liter of petroleum could be realized by 2010 if 20% of Japan's energy consumption could be supplied from gas produced from biomass. It is estimated that a $20 billion market would be established.

- **Development of new technology.**
  The development of environmental analysis, bioremediation and biotope technology to measure and detoxify pollutants will result in a $40 billion market by 2010.

To achieve these goals, the report concludes that development of technology to 1) produce artificial microorganisms with the minimum genome required for growth and to carry out the required process, 2) screening for super enzymes and super microorganisms with unique properties and 3) construct gene libraries from microorganisms that grow under extreme conditions, will be required.

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AMANO ENZYME INC. (Publisher)

Head Office:
2-7, 1-chome
Nishiki, Naka-Ku, Nagoya,
460-8630 Japan
Tel: +81-(0)52-211-3032
Fax: +81-(0)52-211-3054
E-mail: info@amano-enzyme.co.jp

Tokyo Office:
1-1, 1-chome
Uchisaiwai-cho,
Chiyoda-ku, Tokyo
100-001 Japan
Tel: +81-(0)3-3597-0521
Fax: +81-(0)3-3597-0527

AMANO ENZYME U.S.A. CO., LTD.
1157 North Main Street
Lombard, IL 60148-1360, U.S.A.
Tel: +1-630-953-1891
+1-800-446-7652
Fax: +1-630-953-1895
E-mail: aeu@amanoenzymeusa.com

AMANO ENZYME EUROPE LTD.
Roundway House, Cromwell Park, Chipping Norton,
Oxfordshire, OX7 5SR, U.K.
Tel: +44-(0)1608-644677
Fax: +44-(0)1608-644336
E-mail: sales@amano.co.uk

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