Chapter 13

Recombinant DNA Technologies in Food

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Chapter Outline

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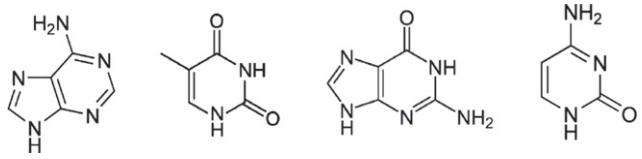
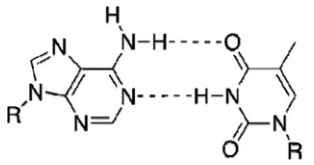
I. INTRODUCTION

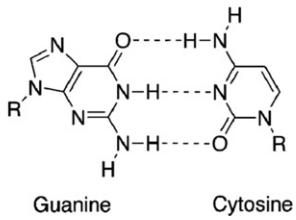
Genetic modification has been used by humans for at least 10,000 years through selective breeding methods of crops, animals, and microorganisms to achieve higher disease resistance, yields, and food quality. During the past 25–30 years, developments in biotechnology have enabled selected traits to be transferred within a species or from one species to another in a much shorter time, using molecular biology techniques manipulating the blueprint of life, deoxyribonucleic acid (DNA).

DNA encodes all the information needed to create an organism. It is the genetic material found mainly in the nucleus of eukaryotic cells. DNA contains four nitrogen bases: adenine (A), thymine (T), guanine (G), and cytosine (C):



All DNA is made up of a backbone of deoxyribose molecules, which are connected by phosphate groups and have nitrogen bases attached to them. The nitrogen bases are found in pairs, with A&T and G&C paired together:





The nitrogen bases are identical in all organisms, but the arrangement of sequence and number of bases creates enormous diversity. Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar, and phosphate are called a nucleotide. Through the backbone of deoxyribose molecules linked by phosphate groups, DNA forms a 'double-helix' structure (Figure 13.1).

Each DNA sequence that occupies a specific location on a chromosome and determines a particular characteristic in an organism is defined as a gene. The size of a gene may vary greatly, ranging from about 1000 bases to 1 million bases in humans. The DNA codes for the amino acid sequence in proteins or other non-protein coding sequences essential for the regulation of the genome. The DNA is transcribed into messenger ribonucleic acid (mRNA), which is

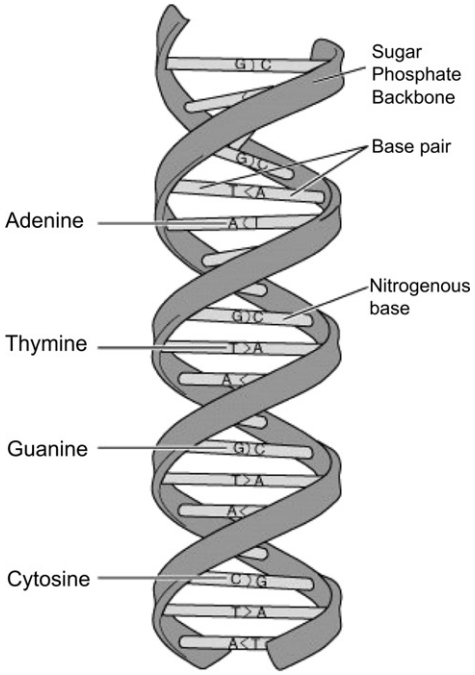


FIGURE 13.1 Double-helix structure of DNA. Supported by a backbone of alternating phosphate and sugar residues, the complementary nucleobases adenine-thymine or guanine-cytosine bind to form a helical structure. *(Source: US National Library of Medicine.)*

a single-stranded molecule of RNA that is synthesized in the cell's nucleus using the genomic DNA as template and then exported to the cytoplasm, where its genetic code is translated into the amino acid sequences constituting the proteins. In RNA the base thymine is substituted with uracil (U).

Recombinant DNA (rDNA) is a single molecule which has been combined artificially from two or more sources outside the living organisms. This recombinant DNA is intended to be introduced into a new host organism to transfer one or more traits for desirable characteristics, or remove genes for undesirable characteristics. There are many reasons to create recombinant DNA, such as the scientific study of the gene products or insertion of foreign genes into other organisms to alter commercially important traits.

Genetically modified organisms (GMOs) and genetically modified microorganisms (GMMs) can be defined as organisms in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination. The resulting organism is said to be genetically modified, genetically engineered, or transgenic. The technology is often called modern biotechnology, genetic engineering, or recombinant DNA technology. The techniques to produce recombinant DNA are routine laboratory procedures with highly specific outcomes. Modern recombinant genetic engineering techniques can be used to transfer genes from microorganisms, plants, or animals into cells from each of these living forms.

Genetic modifications of food have seen a rapid increase in the past decade. The advent of recombinant DNA technology and the possibility of gene transfer between organisms of distinct species, or even distinct phylogenic kingdoms, opened a wide range of recombinant DNA application in food technology. A lot of the food that we eat today contains genetically modified ingredients or is produced with the help of genetic engineering. Foods can consist of or contain living/viable transgenic organisms, e.g. maize, soybeans, and yeast cultures. Foods can contain ingredients derived from GMOs, e.g. flour, food protein products, or oil from genetically modified crops. Single ingredients used in food processing or as food additives can be produced by GMMs, e.g. enzymes and proteins.

The main rationale for the application of transgenic technology in food materials is to overcome the limitations of conventional breeding and selection. Whereas methods of cross-breeding and selection have significantly increased crop, livestock, and even microbial yields over many centuries, the future potential of these methods is constrained by the limitations of sexual-compatibility boundaries between species. Recombinant DNA technology overcomes the limitation of species incompatibility by selectively transferring DNA sequences encoding specific genetic traits (e.g. insect resistance, enhanced growth, better enzyme activity) into a recipient organism so that it expresses this trait. These genetically engineered products have a substantial presence in the food chain where, specifically, genetically engineered microorganisms and plants have a significant commercial value. Therefore, this chapter will

focus on the commercialized applications of genetically engineered crops and microorganisms found in food. Owing to the lack of commercialized genetically modified livestock products, these will not be discussed.

II. GENETICALLY MODIFIED CROPS

Commercial transgenic crops were first planted in 1996 and are now soundly integrated into the agricultural system. The growing area expanded from 1.7 million hectares in 1996 to 148 million hectares worldwide in 2010. The accumulated hectarage from 1996 to 2010 exceeds one billion hectares, indicating the broad acceptance of the new crop traits. These so-called biotech crops are contributing significantly to food security, self-sufficiency, sustainability, alleviation of poverty and hunger, and mitigation of climate change; and the potential of biotech crops for the future is regarded as significant. Biotech crops are now legally planted in 29 countries, where they have an impact on more than half the world's population; hence, they are a significant element of the food chain. This section will give a brief introduction to transgenic crop technology and summarize the genetics and biochemistry for the most important commercially available transgenic crops (James, 2010).

A. Genetic Transformation of Crop Plants

For the process of transgenic plant production several components need to be established:

- A reliable tissue culture and regeneration systems must exist.
- Effective genetic constructs must be created using suitable vectors.
- Efficient transformation techniques for crop plants can be applied.
- Transgenic plants can be recovered and multiplied.
- Stable gene expression can be characterized.
- Trans-genes can be transferred into elite cultivars by conventional breeding.
- Transgenic plants will be evaluated for their effectiveness and environmental hazard (Birch, 1997; Sharma *et al.*, 2004).

Although several approaches have been tried successfully for recombinant DNA integration into crops (Potrykus, 1991), only four approaches are used widely to introduce genes into crop plants (Dale *et al.*, 1993):

- *Agrobacterium*-mediated gene transfer
- microprojectile bombardment with DNA (biolistics)
- microinjection of DNA into the cell nucleus
- direct DNA transfer into isolated protoplasts.

Agrobacterium tumefaciens-mediated gene transfer has been used widely for transformations of crop plants. *Agrobacterium tumefaciens* is a soil-inhabiting bacterium that has been implicated in gall formation at the wound sites in many dicotyledonous plants. This tumor-inducing capability is due to the presence of a large *Ti* (tumor-inducing) plasmid in virulent strains of *Agrobacterium*. Likewise, *Ri* (root-inducing) megaplasmids are found in virulent strains of *Agrobacterium rhizogenes*, the causative agent of 'hairy root' disease. The *Ti* and *Ri* plasmids, and the molecular biology of crown gall and hairy root induction, have been studied in great detail (for further reference see Zambryski *et al.*, 1983; Zambryski, 1992). *Agrobacterium*-mediated transformation is brought about by the incorporation of genes of interest from an independently replicating *Ti* plasmid within the *A. tumefaciens* cell, which then infects the plant cell and transfers the *Ti*-DNA containing the gene of interest into the chromosomes of the actively dividing cells of the host plant (Sharma *et al.*, 2004).

In the particle bombardment (biolistics) method, tungsten or gold particle microprojectiles are coated with the DNA to be inserted, and bombarded into cells or tissues capable of subsequent plant regeneration. Acceleration of heavy microprojectiles (0.5−5.0 μm diameter tungsten or gold particles) coated with DNA carries genes into virtually every type of cell and tissue (Klein *et al.*, 1987; Sanford, 1990). The DNA-coated particles enter the plant cells, the DNA is incorporated in a small proportion of the treated cells, and the transformed cells are selected for plant regeneration (Sharma *et al.*, 2004).

Genetically engineered DNA can also be directly injected into nuclei of embryogenic single cells, which can be induced to regenerate plants in cell culture (Neuhaus *et al.*, 1987). This requires micromanipulation of single cells or small colonies of cells under the microscope, and precise injection of small amounts of DNA solution with a thin

glass micropipette. Injected cells or clumps of cells are subsequently raised in *in vitro* culture systems and regenerated into plants (Sharma *et al.*, 2004).

In the protoplast transformation, the cell wall of the target cells is removed by enzymatic treatment, and the cells are bounded by a plasma membrane (Zhang and Wu, 1988). The DNA can be added into cell suspension, which can be introduced by affecting the plasma membrane with polyethylene glycol or by passing an electric current through the protoplast suspension. The DNA is incorporated into the genome of a few cells. A suitable marker should also be inserted to select the transformed protoplasts and the cell colonies that develop from them (Sharma *et al.*, 2004).

B. Effective Gene Expression in Crop Plants

Efficient genetic engineering relies on the ability to generate a specific gene product at the desired level of expression, in the appropriate tissues, at the right time. This can be accomplished by creating genetic expression constructs (also called expression cassettes) that include promoters and/or transcription regulation elements that control the level, location, and timing of gene expression. A typical expression cassette might include 5′ and 3′ flanking regions homologous to genetic host sequences, enabling integration of the cassette by homologous recombination. A promoter will determine the strength, and temporal and spatial distribution of the open reading frames' expression. The open reading frame contains the code for the heterologous protein. A terminator element regulates proper termination of the RNA's transcription:

A major constraint in the development of effective transgenic products has been the lack of promoters that can offer a high level of gene expression at a high degree of specificity in the crop species of interest. Traditionally, transgene expression has been driven by strong constitutive promoters such as cauliflower mosaic virus *35S* promoter (*CaMV35S*) (Benfey and Chua, 1989, 1990) and *actin 1* (McElroy *et al.*, 1990). Although *CaMV35S* has been widely used in a number of dicotyledonous plant transformation systems, it has low activity in monocotyledonous systems (Wilmink *et al.*, 1995). Moreover, the pattern of *CaMV35S* promoter activity in different tissues of transgenic plants is difficult to predict (Benfey and Chua, 1990). In general, it has been found that monocot promoters are more active in monocot tissues than in dicot tissues (Wilmink *et al.*, 1995; Sharma *et al.*, 2004).

More recently, tissue-specific promoters have been successfully used for driving transgene expression solely in pith tissue. Phosphoenolpyruvate carboxylase (*PEPC*) regulative elements from maize can be used to direct gene expression specifically to green tissue (Hudspeth and Grula, 1989). From the perspective of potential crop yield, many transgenes should be expressed only in those organs where they are needed. For example, insect-resistant traits should be expressed only in those organs likely to be attacked by the insects. Otherwise, plants may be highly resistant, yet the metabolic cost may substantially reduce the crop yield. Tissue-specific expression also reduces the probability of unexpected negative effects on non-target organisms. It seems to be impossible to extrapolate results on gene expression levels from one species to another, and each crop needs be tested with a set of promoters to optimize results. Although the constitutive promoters such as *CaMV35S* are effective in providing high levels of gene expression, such expressions in some cases are not only unnecessary, but could also have unanticipated negative consequences towards non-target organisms. A more targeted expression of insecticidal genes by using tissue- and organ-specific promoters can form an important component for developing transgenic plants with resistance to insects (Wong *et al.*, 1992; Svab and Maliga, 1993; McBride *et al.*, 1995; Sharma *et al.*, 2004).

C. Genetically Modified Crops in the Food Chain

As of September 2011, there were 194 transgenic events for plants/crops approved worldwide for planting, or for use in animal feed or human foods. Twenty-four species, most of them suitable for consumption, were genetically modified to express a variety of traits, mainly herbicide tolerance and insect resistance (ISAAA GM Approval Database). Genetically engineered plants, also called biotech crops, are common commodities in today's agriculture and therefore an essential component of the food chain. In 2010, 148 million ha of biotech crops were grown in 29 countries, representing 10% of all 1.5 billion hectares of cropland in the world. The global worth of this seed alone was valued at US $11.2 billion in 2010, with commercial biotech maize, soybean grain, and cotton valued at approximately US $150 billion per year (James, 2010; Gatehouse *et al.*, 2011).

The advances in transgenic crop modification are often referred to as the 'green revolution'. A significant number of genetically modified food crop varieties gain higher yield and resistance to diseases and pests in many developed and developing countries. However, the resulting intensification and expansion of agriculture bring new forms of health and environmental challenges, for example, increased use of agrochemicals and intensified cultivation resulting in soil erosion. This highlights the fact that we are at the beginning of this green revolution and every genetic modification should be thoroughly evaluated for sustainability through systematic research (James, 2010; Gatehouse *et al.*, 2011).

Insect resistance and herbicide tolerance are the transgenic traits with the highest impact and acceptance on agriculture to date and account for over 99% of global GM crop area (James, 2010; Gatehouse *et al.*, 2011). In addition, there are limited varieties exhibiting virus resistance, delayed ripening, altered nutrient contents, medical applications, improved processing, and altered plant fertility. In the process of genetic engineering, traits establishing resistance to the antibiotics ampicillin, kanamycin, and streptomycin have been cointroduced to allow for the selection of agrobio traits.

D. Insect-Resistant Genetically Modified Crops

1. Bacillus thuringiensis *Toxin-Expressing Crops*

Crops containing *Bacillus thuringiensis* (*Bt*) toxins are currently the only commercialized insect-resistant genetically modified crops. Plants producing *B. thuringiensis* toxin are one of the dominant genetically engineered crops grown on a large scale and in many regions of the world (James, 2009; Then, 2010). Their development progressed rapidly from early genetically engineered tobacco plants in 1985 (Höfte *et al.*, 1986; Vaeck *et al.*, 1987). In 1995, potato plants producing *B. thuringiensis* toxin were approved as safe by the US Environmental Protection Agency (EPA), making them the first pesticide-producing crop to be approved in the USA. Since 1996, *Bt* maize, *Bt* potato, and *Bt* cotton have been grown by farmers in the USA (James, 2010).

Bacillus thuringiensis is a spore-forming pathogenic bacterium distinguished from other members of the *Bacillus* group by its production of crystalline inclusions known as Cry δ-endotoxins. The insect-resistant transgenic crops first commercialized in the mid-1990s expressed all genes encoding entomocidal δ-endotoxins from *B. thuringiensis*, also known as Cry proteins (Soberón *et al.*, 2010; Zhang *et al.*, 2011; Gatehouse *et al.*, 2011). The concept of using Cry proteins was not novel as *B. thuringiensis* spray formulations have been used commercially for approximately four decades to control insect pests, and in particular Lepidoptera (Cannon, 1996). Individual *Bt* toxins have defined insecticidal activity, usually restricted to a few species in one particular order of Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils), Hymenoptera (wasps and bees), and nematodes (de Maagd *et al.*, 2001). Early commercial varieties of insect-resistant transgenic crops expressed single Cry proteins with specific activity against lepidopteran pests, such as cotton expressing Cry1Ac or maize expressing Cry1Ab. Subsequently, other lepidopteran-active *Bt* toxins, such as Cry1F and Cry2Ab2, were introduced and often presented as pyramided genes in a single variety (cotton expressing both Cry1F + Cry1Ac, or cotton expressing Cry1Ac + Cry2Ab2). Cry3 toxins with activity against coleopteran pests are also being used in commercial transgenic crops, particularly maize, to protect against chrysomelid rootworms (e.g. maize expressing Cry3Bb1, maize expressing Cry34Ab1 and Cry35Ab1, and maize expressing a modified version of Cry3A). More recently released transgenic maize varieties express genes encoding Cry proteins active against Lepidoptera and Coleoptera. In China, *Bt* cotton cultivars expressing Cry1Ac together with a modified cowpea trypsin inhibitor (CpTI), which enhances effectiveness, were commercially released in 2000 (Gatehouse *et al.*, 2011).

2. *Cry Proteins: Mode of Action*

Even though Cry toxins have been extensively used commercially, the specifics of their mode of action are still controversial; however, a multistep toxicity process is highly probable (Figure 13.2). The toxicity of *Bt* toxins in target organisms depends on very specific factors such as intestinal pH, proteases, and receptors (Oppert, 1999; de Maagd *et al.*, 2001).

In the spores of *B. thuringiensis* the Bt toxins are produced as inactive crystalline protoxins. Upon ingestion by a susceptible insect larva, the alkaline midgut environment promotes solubilization of crystalline inclusions, releasing the protoxins. Subsequent cleavage by gut proteases at the amino- and carboxyl-terminal ends generates a 65−70 kDa truncated protein, the active δ-endotoxin (Höfte and Whiteley, 1989).

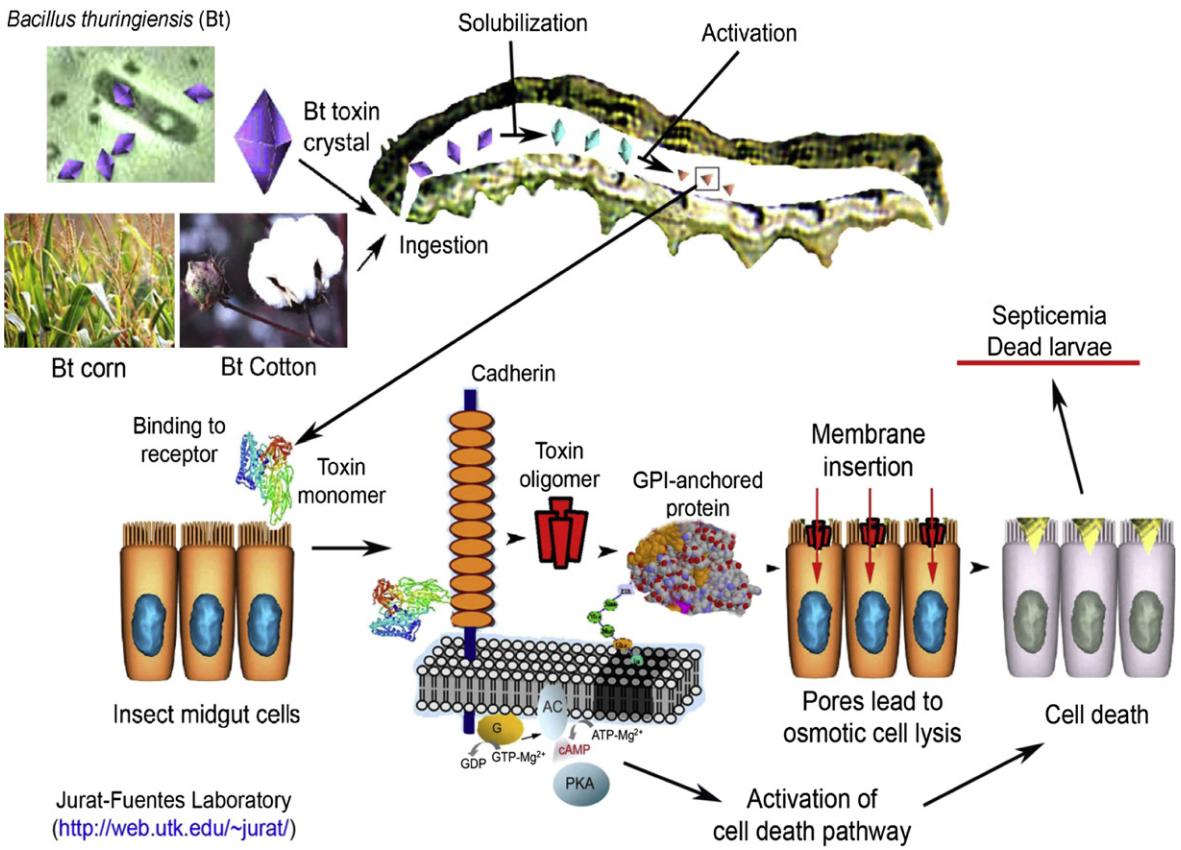


FIGURE 13.2 Suggested mode of action of Cry proteins. The multistep process of Cry toxicity includes ingestion by the insect, solubilization of the proteins, activation by peptide cleavage, binding to the specific cellular receptor catherin and GPI-anchored proteins, which results in activation of cell death pathways and/or oligomerization and pore formation. The pores will allow the release of intracellular contents resulting in cell lysis. *(Source: Jurat-Fuentes Laboratory: http://web.utk.edu/~jurat/.)*

The proteolytically activated toxin binds to receptors (some cadherins have been identified) located on the apical microvillus membrane of epithelial midgut cells (de Maagd *et al.*, 2001; Soberón *et al.*, 2010). Toxin binding to cadherin proteins results in activation of an oncotic cell death pathway and/or formation of toxin oligomers that bind to Glycosylphosphatidylinositol (GPI)-anchored proteins in regions of the cell membrane called lipid rafts (Figure 13.2). Receptor binding causes a conformation change, allowing the proteins' insertion into the cell membrane (Vadlamudi *et al.*, 1995). In the membrane environment several proteins oligomerize to form a pore that leads to osmotic cell lysis followed by death of the insect (de Maagd *et al.*, 2001; Likitvivatanavong *et al.*, 2011; Bravo *et al.*, 2011). Whether oncosis, pore formation, or both mechanisms are ultimately responsible for enterocyte death is still controversial (Figure 13.2). For more information on specific binding refer to Soberón *et al.* (2010) and Likitvivatanavong *et al.* (2011).

In most commercial crop varieties, the Cry proteins are expressed in the active form and as such differ from those used in biopesticide formulations where the Cry proteins are present as protoxins (Gatehouse *et al.*, 2011).

The significant future potential of these proteins is demonstrated by the fact that 634 different variations of toxins have so far been identified in diverse strains of *B. thuringiensis* (Crickmore, 2011).

3. Bacillus thuringiensis *Toxins: Environmental and Health Impact*

Owing to the specific intrinsic factors, such as pH, specific proteases, and specific receptor binding required for activation, the effects of *Bt* toxins are limited to the target insects, with little or no impact on humans, wildlife, pollinators, and most other beneficial insects (Gill *et al.*, 1992; Oppert, 1999; de Maagd *et al.*, 2001). The currently commercialized *Bt* toxins do not exhibit acute toxicity in mammals. Because no toxic effects of *Bt* toxins in humans have been detected in its 70 years of use, it is now considered an acceptable pest-control measure for the organic food industry (Whalon and Wingerd, 2003).

4. *Commercial* Bacillus thuringiensis *Crops*

Bacillus thuringiensis Cry proteins are being integrated in an ever-increasing variety of commercial corps, as demonstrated in Table 13.1.

TABLE 13.1 Selection of Commercial Transgenic Crops

Common Name of Food Product	Common Name of Parent Organism and OECD Unique Identifier	Details of Modified Trait	Gene(s) Responsible for Modified Trait
Corn			
Corn oil, flour, sugar or syrup	Yieldgard corn (MON810)	Insect protected	*cry 1A(b)* gene from *B. thuringiensis* subsp. *kurstaki*
	MON-00810-6		
Corn oil, flour, sugar or syrup	Bt-176 corn	Insect protected	*cry1A(b)* gene from *B. thuringiensis* subsp. *kurstaki*
	SYN-EV176-9		
Corn oil, flour, sugar or syrup	Bt-11 corn	Insect protected and glufosinate-ammonium tolerant	*cry1A(b)* gene from *B. thuringiensis, pat gene derived from the bacteria Streptomyces viridochromogenes*
	SYN-BT011-1		
Corn oil, flour, sugar or syrup	Bt – Liberty Link Corn line DBT418	Insect protected and glufosinate-ammonium tolerant	Gene for CryIAc from *B. thuringiensis*. Gene for Bar is derived from *Streptomyces hygroscopicus*
	DKB-89614-9		
Corn oil, flour, sugar or syrup	Herculex Insect Protection	Protected from major lepidopteran insect pests, including the European corn borer, and glufosinate-ammonium tolerant	*cry1F* gene is a synthetic version of a gene from *B. thuringiensis*. The *pat* gene is derived from *Streptomyces viridochromogenes*
	DAS-01507-1		
Corn oil, flour, sugar or syrup	Insect Resistant MON863 corn	Protected against corn rootworm	*cry3Bb1* variant derived from *B. thuringiensis*
	MON-00863-5		
Corn oil, flour, sugar or syrup	Bt Cry34/35Ab1 corn	Corn rootworm protection and glufosinate-ammonium tolerant	*cry34Ab1* and *cry35Ab1* genes from *B. thuringiensis* and the *pat* gene from *Streptomyces viridochromogenes*
	DAS-59122-7		
Foods derived from corn, e.g. corn syrup, flour, and oil	MIR604 corn	Corn rootworm protected	A modified *Cry3A* gene from *B. thuringiensis*. Also contains the *pmi* gene from *E. coli* (encodes phosphomannose isomerase, a selectable marker)
	SYN-IR604-5		
Foods derived from corn, e.g. corn syrup, flour, and oil	Roundup ready, insect protected corn MON88017	Glyphosate tolerance and corn rootworm protected	*Cry3Bb1* from *B. thuringiensis* and *cp4 EPSPS*, derived from *Agrobacterium* species strain *CP4*
	MON-88017-3		
Cotton			
Cotton oils and linters	Ingard® cotton	Insect protected	*cry1Ac* gene from *B. thuringiensis*

TABLE 13.1 Selection of Commercial Transgenic Crops—cont'd

Common Name of Food Product	Common Name of Parent Organism and OECD Unique Identifier	Details of Modified Trait	Gene(s) Responsible for Modified Trait
	MON-00531-6		
	MON-00757-7		
	MON-89924-2		
Cotton oils and linters	Bollgard II® cotton	Insect protected	*cry1Ac* and *cry2Ab* genes from *B. thuringiensis*
	MON-15985-7		
Cottonseed oils and linters	COT102 cotton	Insect protected	*vip3a* gene from *B. thuringiensis* subsp. *kurstaki* and *hph* gene (hygromycin resistance) from *E. coli*
	SYN-IR102-7		
Cottonseed oils and linters	MXB-13 cotton	Insect-protected and glufosinate-ammonium tolerant	*cry1Ac* and *cry1F* genes from *B. thuringiensis* subsp. *kurstaki* and *aizawai*, respectively, and the *pat* gene from *Streptomyces viridochromogenes*
	DAS-24236-5 × DAS-21023-5		
Potatoes			
Potatoes	New Leaf® potatoes	Protected against a range of insects, including the Colorado potato beetle	*cry3Aa* gene bacterium *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*)
	NMK-89812-3		
	NMK-89170-9		
	NMK-89879-1		
	NMK-89576-1		
Potatoes	New Leaf® Plus potatoes	Protection against CPB and PLRV	*cry3Aa* gene from the soil bacterium *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) and *PLRVrep* gene from PLRV
	NMK-89185-6		
	NMK-89684-1		
	NMK-89896-6		
Potatoes	New Leaf® Y potatoes	Protected against a range of insects, including CPB, and protected against potato virus Y	*cry3Aa* gene from the soil bacterium *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) and coat protein gene (*PVYcp*) from potato virus Y
	NMK-89653-6		
	NMK-89935-9		
	NMK-89930-4		
Soybean			
Soy foods, soybean oil or protein meal	MON-87701-2	Insect protected from lepidopteran larvae	*cry1Ac* gene from *B. thuringiensis* subsp. *kurstaki*

CPB: Colorado potato beetle; PLRV: potato leafroll virus; *B. thuringiensis*: *Bacillus thuringiensis.*

E. Herbicide-Tolerant Crops

From 1996 to 2010, herbicide-tolerant crops consistently occupied the largest planting area of biotech crops. In 2010 alone, herbicide-tolerant crops occupied 89.3 million hectares or 61% of the 148 million hectares of biotech crops planted globally. In 2010, 93% of all soybeans grown in the USA were herbicide resistant, as were 78% of all cotton and 70% of all maize varieties (USDA, 2011). The most common are the glyphosate- and glufosinate-tolerant varieties.

Many herbicides kill plants by interfering with enzyme functions in the plant. Most herbicides exert their effect on a single enzyme which catalyzes a key metabolic reaction in the plant. In general, plants exhibit a range of sensitivities to the herbicides used in agriculture, with some species exhibiting considerable tolerance to a single herbicide. There are several mechanisms by which plants can tolerate exposure to herbicides:

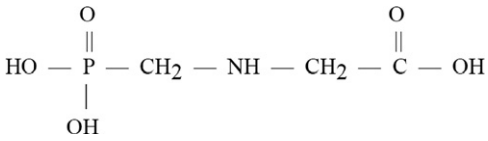
- The plant produces an enzyme which detoxifies the herbicide.
- The plant produces an altered target enzyme which is not affected by the herbicide.
- The plant produces physical or physiological barriers to uptake of the herbicide into the plant tissues and cells (OECD, 1999; Devine and Shukla, 2000).

Strategies to engineer herbicide tolerance into crops focus on the first two mechanisms. Transgenes are introduced to express enzymes detoxifying the herbicide or to replace the intrinsic herbicide-target enzyme with a variant resistant to the inhibition.

This section will summarize the information available on the source of the genes that have been used to construct herbicide-tolerant transgenic plants and the nature of the enzymes they encode and their role in the plants' metabolism. It will not discuss the wealth of information available on the herbicides or the uses of the herbicide in agricultural and other applications. Food safety aspects of the use of herbicide-tolerant transgenic plants will not be discussed extensively. Additional information on aspects not discussed is available from other sources, including the respective governmental organizations that regulate the use of the herbicides.

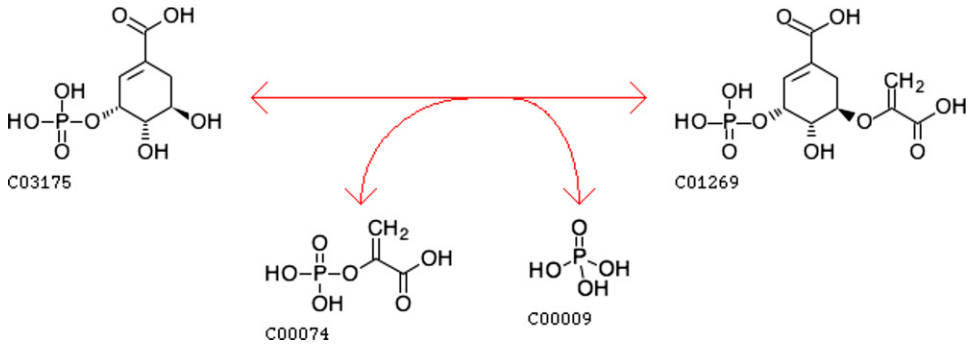
1. Tolerance to the Herbicide Glyphosate

Glyphosate (*N*-phosphonomethyl-glycine) is the most widely used broad-spectrum weed control agent with minimal human and environmental toxicity (Duke and Powles, 2008; Pollegioni *et al.*, 2011). It is not a natural product – it is derived by chemical synthesis – and it is the active ingredient of the herbicide Roundup® (Monsanto):



Glyphosate is a highly specific reversible competitive inhibitor of the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS, EC 2.5.1.19) which catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate 3-phosphate (S3P) to produce 5-enolpyruvyl shikimate 3-phosphate (EPSP) and inorganic phosphate (KEGG R03460):

Phosphoenolpyruvate + Shikimate 3-phosphate <=> Orthophosphate + 5-*O*-(1-Carboxyvinyl)-3-phosphoshikimate



In this reaction glyphosate competes with phosphoenolpyruvic acid, but it does not inhibit any other phosphoenolpyruvic acid-dependent enzymatic reactions. This reaction forms the sixth step in the shikimate pathway leading to the synthesis of aromatic amino acids and other aromatic compounds in plants, fungi, bacteria, and apicomplexan parasites. The following (Figure 13.3) graph illustrates the shikimate pathway that leads to the biosynthesis of aromatic amino acids, and the mode of action of glyphosate on the reaction catalyzed by EPSPS (Pollegioni *et al.*, 2011):

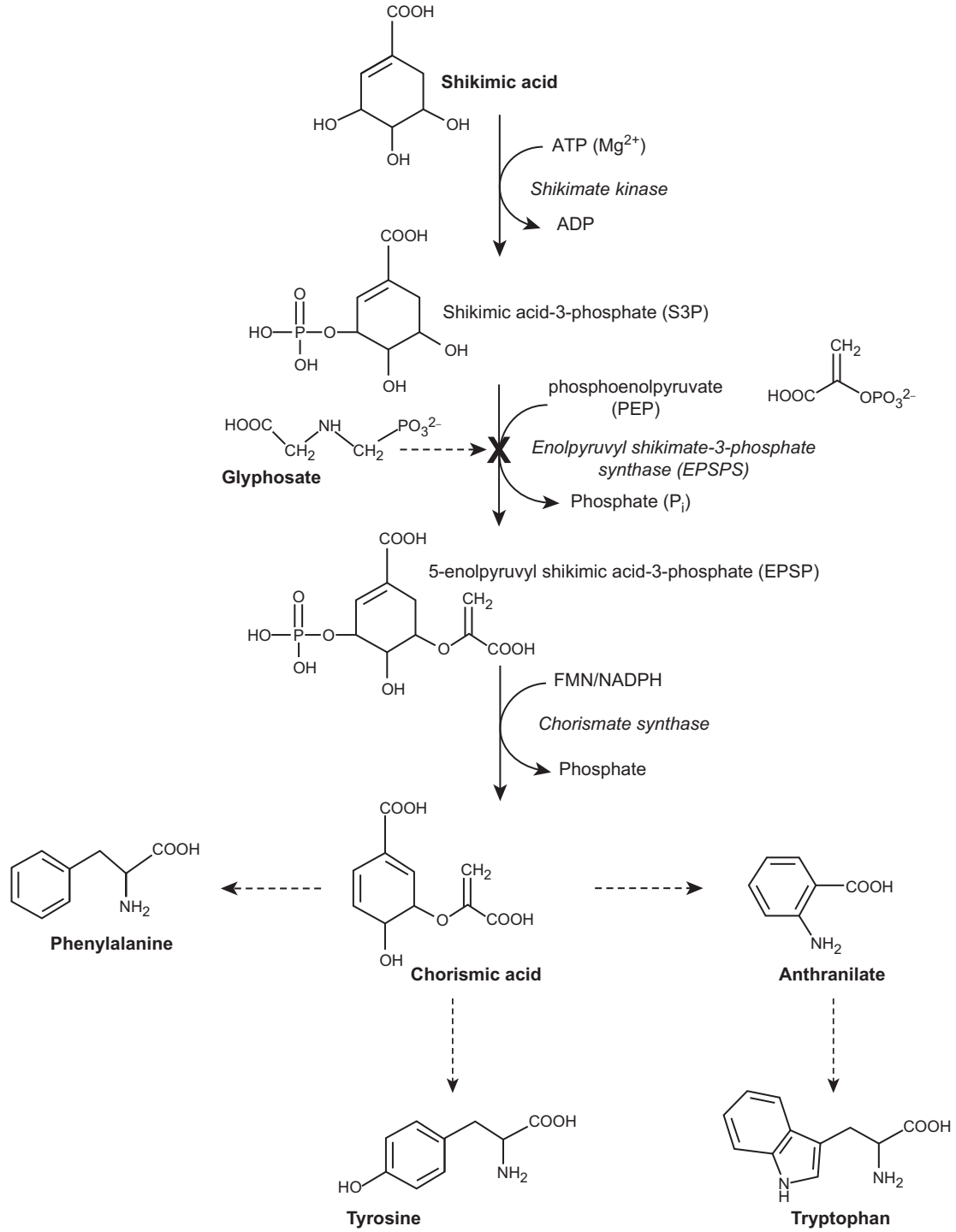


FIGURE 13.3 The shikimate pathway of aromatic amino acids biosynthesis and its inhibition by glyphosphate, which acis on the enzyme enolpyruvylshikimate-3-phosphate synthase *(Pollegiotti et al., 2011).*

As a consequence of the inhibition of aromatic amino acid biosynthesis, protein synthesis is disrupted, resulting in the plant's death (Kishore and Shah, 1988). No orthologs of the *EPSPS* gene are found in animals (Steinrucken and Amrhein, 1980).

The high sensitivity of crop plants to glyphosate has limited its use as a pre-crop emergence herbicide in no-till management strategies, and as a herbicide and crop desiccant when applied shortly before crop harvest. Traditional mutagenesis and selection techniques have failed to produce a useful level of tolerance in crop plants (OECD, 2010). With the development of genetically engineered crop plants that are resistant to glyphosate, this herbicide can be applied after both crops and weeds have emerged, with little or no damage to the crop (James, 2009).

Based on the knowledge of the mode of action of glyphosate, two major transgenic strategies have proven successful to confer herbicide tolerance, i.e. to produce glyphosate-tolerant plants: introduction of a glyphosate-tolerant EPSPS enzyme and introduction of the glyphosate-inactivating enzyme, glyphosate oxidoreductase (GOX). Recombinant DNA techniques have been used to express genes that encode glyphosate-tolerant EPSPS enzyme alone or a combination of EPSPS and GOX genes in susceptible plants (Nida *et al.*, 1996; Padgette *et al.*, 1995, 1996).

2. Glyphosate-Tolerant 5-Enolpyruvyl-3-Phosphoshikimic Acid Synthase Transgenes

The revolutionary new glyphosate use pattern commenced in 1996 with the introduction of a transgenic glyphosate-resistant soybean, launched and marketed under the Roundup Ready brand in the USA. Two EPSPS genes which provide field-level tolerance to glyphosate have been introduced into commercial cultivars.

Agrobacterium sp. CP4, isolated from a waste-fed column at a glyphosate production facility, yielded a glyphosate-resistant, kinetically efficient EPSPS (the so-called CP4 EPSPS) that is suitable for the production of transgenic, glyphosate-tolerant crops. The CP4 enzyme has unexpected kinetic and structural properties that make it unique among the known EPSPSs, and it is therefore considered to be a prototypic class II EPSPS (for further information see Pollegioni *et al.*, 2011). An intriguing feature is the strong dependence of the catalytic activity on monovalent cations, namely K^+ and NH_4^+. The lack of inhibitory potential ($K_i > 6$ mM) is primarily attributed to alanine (Ala)^{100} and leucine (Leu)^{105} in place of the conserved plant residues glycine (Gly)^{96} and proline (Pro)^{101} (Figure 13.3). The presence of Ala^{100} in the CP4 enzyme is of no consequence for the binding of phosphoenolpyruvic acid, but glyphosate can only bind in a condensed, high-energy, and non-inhibitory conformation. Glyphosate sensitivity is partly restored by mutation of Ala^{100} to glycine, allowing glyphosate to bind in its extended, inhibitory conformation (Pollegioni *et al.*, 2011).

When the *Agrobacterium* EPSPS is present in transgenic plants it realizes the aromatic amino acid needs of the plant in the presence of glyphosate, whereas the plant version of this enzyme (ubiquitous in nature) is sensitive to glyphosate. *Agrobacterium* spp. are not human or animal pathogens, but some species are pathogenic to plants (Holt, 1984; Croon, 1996).

Recently, the EPSPS gene from corn (*Zea mays*) has been mutated *in vitro* to obtain a glyphosate-tolerant enzyme. In the tolerant version of the enzyme, called mEPSPS, threonine (Thr)^{102} is replaced by isoleucine (Ile) and proline (Pro)^{106} is replaced by serine (Ser) (Monsanto, 1997). The location of the changed amino acids resembles the situation in the *Agrobacterium* isoform and therefore confers glyphosate tolerance by the mechanism described in Figure 13.4.

EPSPS enzyme is synthesized in the cytoplasm and then transported to the chloroplast (Kishore and Shah, 1988). The translocation of the protein to the chloroplast is carried out by an N-terminal protein sequence called the chloroplast transit peptide. Chloroplast transit peptides are typically cleaved from a mature protein and degraded following delivery to the plastid (Della-Cioppa *et al.*, 1986). A plant-derived coding sequence expressing a chloroplast transit peptide is often linked with each of the genes imparting glyphosate tolerance. This peptide facilitates the import of the newly translated enzymes into the chloroplasts, the site of both the shikimate pathway and glyphosate mode of action (OECD, 2010).

The constitution of the expression cassette for the CP4 EPSPS integrated into the sugar beet line H7-1 illustrates the different genetic elements necessary for optimal expression and targeting of the transgene. It contains a region of T-DNA that is delineated by left and right border sequences, and contains a single *cp4 epsps* gene with essential regulatory elements necessary for expression in the chloroplasts of the sugar beet plants. The organization of the T-DNA, corresponding to approximately 3.4 kb, is depicted in Figure 13.5 (FSANZ A525). The size and function of each of the genetic elements present in the expression cassette are described in Table 13.2 (FSANZ A525).

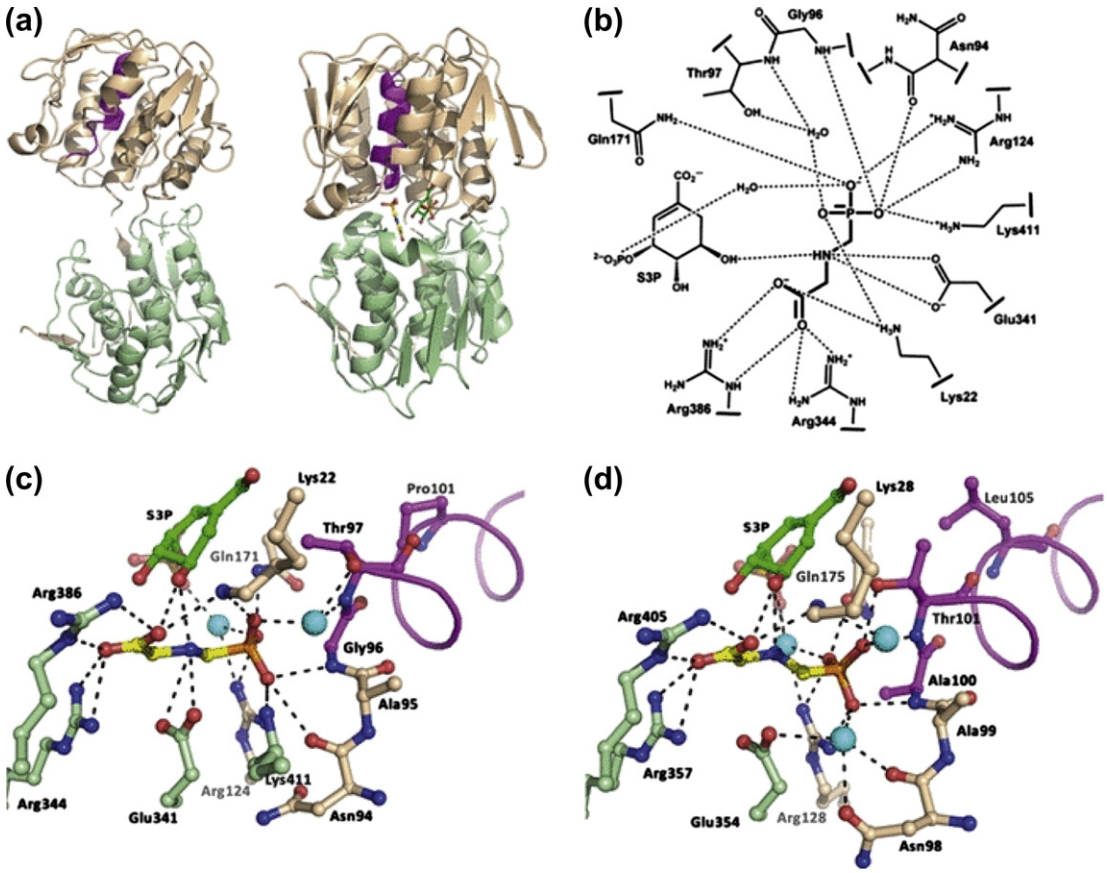


FIGURE 13.4 Molecular mode of action of glyphosate and the structural basis for glyphosate resistance. (a) In its ligand-free state, 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS) exists in the open conformation (left; PDB: 1eps). Binding of S3P induces a large conformational change in the enzyme to the closed state, to which glyphosate or the substrate PEP binds (right, PDB: 1g6s). The respective crystal structures of the E. coli enzyme are shown. (b) Schematic representation of potential hydrogen-bonding and electrostatic interactions between glyphosate and active site residues including bridging water molecules in EPSPS from E. coli (PDB: 1g6s). (c) The glyphosate-binding site in EPSPS from E. coli (PDB: 1g6s). Water molecules are shown as spheres, and the residues known to confer glyphosate resistance upon mutation are Pro^{101}, Thr^{97}, and Gly^{96}. (d) The glyphosate-binding site in CP4 EPSPS (PDB: 2gga). The spatial arrangement of the highly conserved active site residues is almost identical for class I (E. coli) and class II (CP4) enzymes, with the exception of an alanine at position 100 (Gly^{96} in E. coli). Another significant difference is the replacement of Pro^{101} (E. coli) by a leucine (Leu^{105}) in the CP4 enzyme. Note the markedly different, condensed conformation of glyphosate as a result of the reduced space provided for binding in the CP4 enzyme. *(Modified from Pollegioni et al., 2011.)*

In order to achieve efficient gene expression and protein translation of bacterial genes within plants the codon usage needs to be optimized. This has been done for the codons of the *Agrobacterium* glyphosate-tolerant EPSPS gene by chemical DNA synthesis for codon optimization in plants. The amino acid sequence of the resulting enzymes is not changed. In expression cassettes, the gene coding sequences are usually linked to regulatory sequences like promoters, terminators, enhancers, and introns, which are also optimized for plant expression (Parrish *et al.*, 1995).

During the life cycle of any herbicide-tolerant plant, the plant is exposed only rarely to the herbicide. Except for the production of the enzyme(s) encoding glyphosate tolerance, there should be no other changes in plant metabolism. After glyphosate application, the enzyme activities expressed by the transgenes enable the plant to survive herbicide exposure. In the case of introduced EPSPS, no new metabolic products are formed since the only difference from the native enzyme is its insensitivity to glyphosate. However, in situations of very high expression the levels of downstream metabolites might change (OECD, 1999).

Expression of glyphosate-tolerant EPSPS is not detrimental to plant growth, since such crops have agronomic performance similar to their parents. Governmental regulatory agencies in the USA (US Department of Agriculture, 1994, 1995, 1997), Canada (Agriculture and Agrifood Canada, 1995, 1996), Japan (Ministry of Agriculture, Forestry and Fisheries, 1996), and the European Union (European Commission, 1998a, b) have made decisions that the

FIGURE 13.5 Organization of the T-DNA cassette for the CP4 EPSPS gene integrated into the sugar beet line H7-1.

TABLE 13.2 Size and Function of Genetic Elements Present in the Expression Cassette for the cp4 epsps Gene

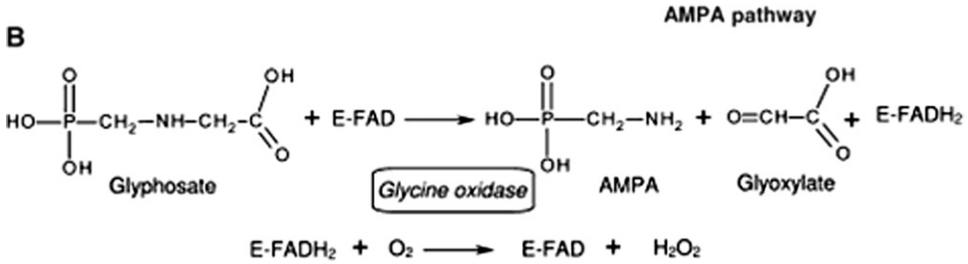
Genetic Element	Size (kb)	Description and Reference
Right border	0.025	A 21–25 bp nucleotide sequence that acts as the initial point of DNA transfer into plant cells, originally isolated from *Agrobacterium tumefaciens* plasmid pTiT37
P-FMV	0.672	The 35S gene promoter from a modified figwort mosaic virus (FMV)
ctp2	0.31	The N-terminal chloroplast transit peptide sequence from the *Arabidopsis thaliana epsps* coding region
cp4 epsps	1.363	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) coding region from *Agrobacterium* sp. strain CP4
E93′	0.63	The 3′ end of the *Pisum sativum rbc*S E9 gene, containing polyadenylation sites that direct mRNA processing and polyadenylation
Left border	0.025	A 21–25 bp nucleotide sequence that delimits the T-DNA transfer into plant cells, originally isolated from *A. tumefaciens* plasmid pTi 15955, a derivative of the octopine type plasmid, pTiA6

presence of the EPSPS protein in plants does not result in plants that are unsafe in their environments. Several lines of evidence support the conclusion that these enzymes show low mammalian toxicity:

- Neither enzyme shows amino acid homology to known allergens or mammalian toxins.
- Data from acute oral toxicity tests at high concentration of enzymes showed no toxicity (Harrison *et al.*, 1996). In acute oral toxicity tests of bacterially derived CP4 EPSPS protein, no adverse effects occurred at a dose of 572 mg/kg body weight of the test animals.
- The enzyme is readily inactivated by heat or mild acidic conditions and is readily degraded in an *in vitro* digestibility assay, which is consistent with the lack of oral toxicity (US EPA, 1996, 1997). This is consistent with the observation that most enzymes are not considered toxic to vertebrates (Kessler *et al.*, 1992).

3. Glyphosate Oxidoreductase

Glyphosate oxidoreductase (GOX) catalyzes the oxidative cleavage of the carbon–nitrogen bond on the carboxyl side of glyphosate, resulting in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate, effectively deactivating the substrate as a herbicide (Pollegioni *et al.*, 2011):



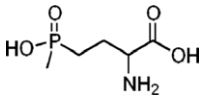
The gene that encodes for the glyphosate oxidoreductase was isolated from *Achromobacter* strain LBAA, a soil bacterium ubiquitous in nature (Barry and Kishore, 1997). GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme.

To facilitate the expression of GOX in plants, the gene sequence was redesigned to eliminate stretches of G and C of five or greater, A + T-rich regions that could function as polyadenylation sites or potential RNA-destabilizing regions, and codons not frequently found in plant genes. When this gene is transfected into plants, expression of GOX results in glyphosate tolerance (Pollegioni *et al.*, 2011).

The presence of GOX is not expected to have an impact on the plant's metabolome, since it only converts glyphosate to AMPA and glyoxylate when glyphosate herbicide is applied. Since aminomethylphosphonic is a naturally occurring plant metabolite involved in carbon cycling, it will be further metabolized to provide intermediates for the Krebs cycle. GOX is highly specific for its substrate, glyphosate; therefore, in the absence of glyphosate no metabolites are expected. The US EPA has decided that only glyphosate residues are to be regulated in plant and animal commodities, and that the major metabolite AMPA is not of toxicological concern regardless of its level in food (US EPA, 1997). Expression of GOX is not detrimental to plant growth, since such crops have agronomic performance similar to their parents. The presence of the GOX protein in plants does not result in plants that are unsafe in their environments or exhibit mammalian toxicity (US EPA, 1997).

4. Tolerance to the Herbicide Glufosinate

Glufosinate [phosphinothricin; DL-homoalanin-4-yl(methyl)phosphinic acid] is a racemic phosphinico amino acid (Hoerlein, 1994):



Its ammonium salt (glufosinate-ammonium) is widely used as a non-selective herbicide and is the active ingredient of many commercial herbicide formulations. The L-isomer of glufosinate is a structural analog of glutamate and, therefore, is a competitive inhibitor of the enzyme glutamine synthetase (GS) of bacteria and plants. The D-isomer is not a glutamine synthetase inhibitor and is not herbicidally active (OECD, 2002).

Owing to the inhibition of glutamine synthetase, non-tolerant plant cells accumulate large amounts of toxic ammonia produced by nitrate assimilation and photorespiration (Tachibana *et al.*, 1986) and the level of available glutamine drops (Sauer *et al.*, 1987). Damage to cell membranes and inhibition of photosynthesis are followed by plant cell death. The action of glufosinate is dependent on environmental conditions. Temperatures below 10°C, as well as drought stress, reduce its efficacy because of the limited metabolic activity of the plant (OECD, 2002).

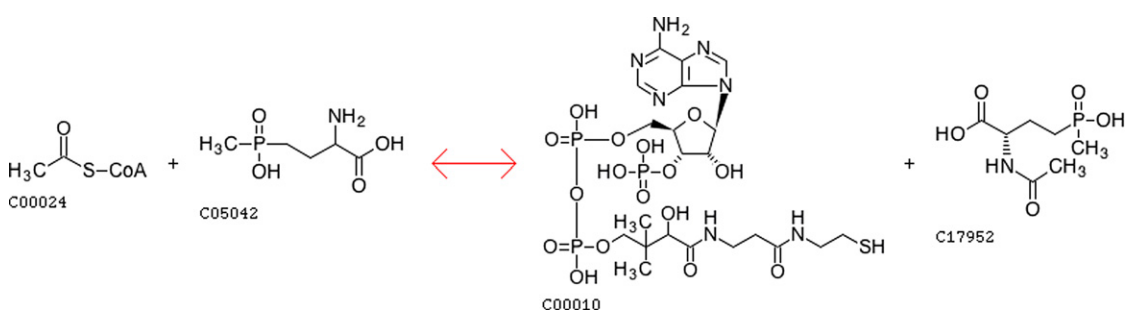
Glufosinate is a contact herbicide and is taken up by the plant primarily through the leaves. There is no uptake from the soil through the roots, presumably because of the rapid degradation of glufosinate by soil microorganisms. There is limited translocation of glufosinate within the plant. After application of L-glufosinate, *N*-acetyl-L-glufosinate and further metabolites on distinct leaves, a preferential transport into the upper leaves and a low level of translocation into the lower plant parts were observed in both genetically modified and unmodified tobacco plants (Dröge-Laser *et al.*, 1994).

Glufosinate has a wide spectrum of activity encompassing monocotyledonous and dicotyledonous species. Because of its limited systemic action, there is no enduring effect on perennial weeds. Weeds emerging after herbicide application are not affected (OECD, 2002).

5. Phosphinothricin Acetyltransferase

Phosphinothricin acetyltransferase (EC 2.3.1.183) catalyzes the acetylation of L-phosphinothricin (KEGG R08938):

Acetyl-CoA + Glufosinate <=> CoA + *N*-Acetylphosphinothricin



The phosphinothricin acetyltransferase (PAT) proteins, which are encoded by the *bar* coding sequence from *Streptomyces hygroscopicus* or the *pat* coding sequence from *Streptomyces viridochromogenes*, are present in glufosinate-ammonium-tolerant plant varieties of various crops such as corn, cotton, rice, oilseed rape, and soybean. The PAT enzymes acetylate L-phosphinothricin, the active isomer of the glufosinate-ammonium herbicide, resulting in tolerance of transgenic plants to postemergent application of the non-selective herbicide (Hérouet *et al.*, 2005).

The native *S. hygroscopicus pat* gene has been resynthesized to modify codon usage for improved protein expression in plant cells. At the nucleotide sequence level, the synthetic gene demonstrates 70% homology with the native *pat* gene from *S. viridochromogenes*. The amino acid sequences of the PAT enzyme encoded by the native and synthetic genes are identical.

Using recombinant DNA technologies, the *bar* or the *pat* coding sequences are each fused to an appropriate promoter and terminator for plant gene expression and introduced into the plant genome.

The *bar* gene is often under the control of the plant promoter (*Pssu-Ara*), which directs expression of PAT predominantly in the green tissues (leaves and stems). Alternatively, in constructs involving the synthetic *pat* gene, the plant viral promoter *P35S* has been used for constitutive expression of the PAT protein in all tissues of the plant.

In genetically modified glufosinate-tolerant plants expressing the PAT enzyme, it appears that two metabolic routes compete:

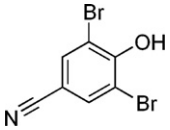
- The deamination of glufosinate and subsequent conversion of 4-methylphosphinico-2-oxo-butanoic acid (PPO) to 3-methylphosphinico-propionic acid (MPP) or to 4-methylphosphinico-2-hydroxy-butanoic acid.
- The N-acetylation of L-glufosinate by PAT (Dröge-Laser *et al.*, 1994). The second of these two routes predominates when PAT specific activity is relatively high (OECD, 2002).

In all glufosinate-tolerant crops, the principal metabolic residues are *N*-acetyl-L-glufosinate, glufosinate-ammonium, and 3-methylphosphinico-propionic acid. Besides these principal residues, trace levels of other metabolites were also identified in soybean including 2-methylphosphinico-acetic acid (MPA) and 4-methylphosphinico-butanoic acid (MPB). The herbicidally inactive D-glufosinate appears to be stable in plants owing to the L-specific acetylation activity of the PAT enzyme (Dröge-Laser *et al.*, 1994).

The *pat* and the *bar* coding sequences as well as their respective PAT proteins are not toxic and do not possess any of the characteristics associated with food allergens. There is a reasonable certainty of no harm resulting from the inclusion of the PAT proteins in human food or in animal feed (Hérouet *et al.*, 2005).

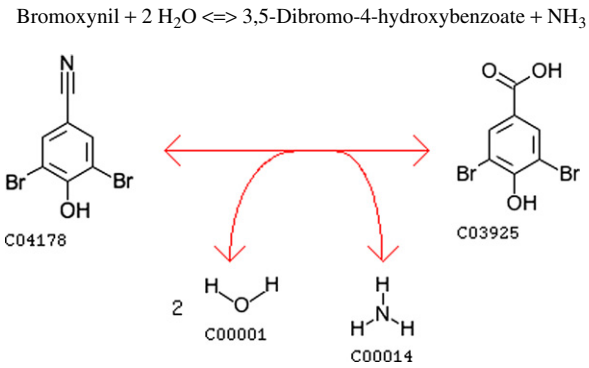
6. Tolerance to the Herbicide Bromoxynil

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) is a systemic herbicide, which is widely used for post-emergence control of annual broadleaved weeds, especially young seedlings of cereals, ryegrass-seed crops, turf, and non-crop land:



It is often used in combination with other herbicides to extend the spectrum of weed control. Bromoxynil inhibits photosynthetic electron transport and also uncouples oxidative phosphorylation in mitochondria, thereby stopping energy production and negatively affecting plant respiration (EPA738-R-98-013). Inhibition of electron transport causes superoxide production, resulting in the destruction of cell membranes and an inhibition of chlorophyll formation, leading to plant death. Bromoxynil octanoic and heptanoic acid esters are also applied as herbicides.

Bromoxynil nitrilase (EC 3.5.5.6) hydrolyzes carbon−nitrogen bonds, other than peptide bonds (KEGG R04349):



This degradation effectively inactivates the herbicide and enables the normally bromoxynil-sensitive plant to survive and grow when treated with applications of the herbicide.

Bromoxynil nitrilase is encoded by the *oxy* gene of the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae* (Stalker and McBride, 1987). When integrated in an expression cassette and transferred into plants, the gene, through its encoded protein, confers tolerance to the oxynil family of herbicides including bromoxynil and ioxynil.

In a typical expression cassette the *oxy* gene is flanked by specific sequences, as found in pBrx75, responsible for the trait in BXN cotton lines (FSANZ A379) (Figure 13.6, Table 13.3).

The 35s cauliflower mosaic virus promoter region ensures strong initiation of translation. The 3′ non-translated region of the *tml* gene from *A. tumefaciens* contains signals for termination of transcription and directs polyadenylation. The gene *nptII* codes for neomycin phosphotransferase II from Tn5 in *Escherichia coli* and confers resistance to the antibiotics kanamycin and neomycin. It is used as a selectable marker for plant transformation (Table 13.3).

F. Male Sterility: The *Barstar* and *Barnase* Gene System

Sterility of transgenic crops can be regulated through the selective expression of two genes called *barnase* and *barstar*, which control the development of pollen.

The *barnase* gene encodes the enzyme barnase, a ribonuclease derived from *Bacillus amyloliquefaciens*, which disrupts the production of RNA. When the *barnase* gene is selectively expressed in the tapetal cell layer of the anther, a cell layer that plays a vital nutritive role during pollen formation, it destroys the tapetal cell layer, rendering the anthers incapable of producing viable pollen grains. This inability to produce viable pollen grains renders the plant male sterile and provides reliable pollination control (FDA BNF No. 000031).

Fertility is restored by the expression of the *barstar* gene, also originating from *B. amyloliquefaciens*, coding for an inhibitor of the barnase protein. When the barnase protein is expressed in the tapetum cells it leads to the restoration of fertility by tightly binding and inactivating the barnase. The coexpression is achieved through traditional breeding of two separate crop lines, each carrying one of the two genes. Plant lines expressing *barstar* are referred to as fertility restorers.

Thus, the hybrid system consists of crossing a male sterile line (female parent) with a specific fertility restorer line, giving rise to progeny that are fully fertile. The primary objective of these modifications is the production of a range of parental lines with superior agronomic performance that are to be used in a breeding system for producing hybrids yielding significantly more seed (FSANZ A372).

G. Pathogen-Derived Virus-Resistant Crops

Plant viruses cause significant crop losses, specifically demonstrated by the effects of a few single viruses for a specific host in a particular geographical region (for reviews, see Bos, 1982; Waterworth and Hadidi, 1998). For

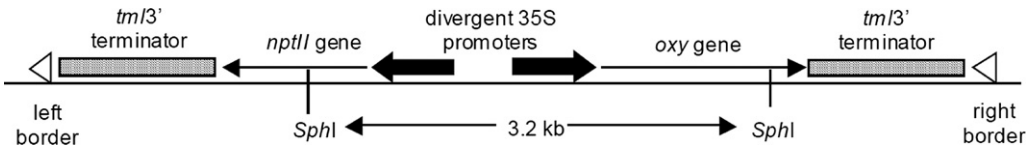


FIGURE 13.6 Expression cassette pBrx75 conferring bromoxynil resistance.

TABLE 13.3 Description of the Elements of Expression Cassettes pBrx75 Conferring Bromoxynil Resistance

Genetic Element	Source	Function
35S promoter	The cauliflower mosaic virus (CaMV) 35S promoter region	A promoter for high level constitutive expression in plant tissues
oxy	Gene isolated from *Klebsiella pneumoniae* subspecies *ozaenae* encoding the enzyme nitrilase	Inactivates the herbicide bromoxynil and confers bromoxynil tolerance when expressed in plants
tml 3.	The 3. non-translated region of the *tml* gene from *Agrobacterium tumefaciens* plasmid pTiA6	Contains signals for termination of transcription and directs polyadenylation
nptII	The gene coding for neomycin phosphotransferase II from Tn5 in *Escherichia coli*	Confers resistance to the antibiotics kanamycin and neomycin. Used as a selectable marker for plant transformation

instance, the annual global impact of *Tomato spotted wilt virus* (TSWV) and the viruses causing African cassava mosaic disease, which are of exceptional agronomic importance, has been estimated to be approximately US $1 billion each (Taylor *et al.*, 2004).

The concept of pathogen-derived resistance (PDR) offers a novel approach to developing virus-resistant crop plants. Resistance is engineered in otherwise susceptible hosts by introducing coding or non-coding DNA sequences from the pathogen's genome (Sanford and Johnston, 1985). For plant viruses, the concept of pathogen-derived resistance was first validated with the development of tobacco expressing the coat protein of *Tobacco mosaic virus* (TMV), which led to diminished or abolished infection (Powell Abel *et al.*, 1986). This breakthrough discovery paved the way for the creation of numerous virus-resistant transgenic plants, including horticultural crops. Crop plants expressing viral genetic elements have been tested successfully in the field and a few have been commercialized. The deployment of virus-resistant transgenic plants has become an important strategy for effective and sustainable control of major virus diseases (Gottula and Fuchs, 2009).

1. Mechanism of Pathogen-Derived Virus Resistance

Resistance to numerous plant viruses was initially engineered by using viral coat protein genes, following the discovery that transgenic tobacco expressing the coat protein gene of TMV exhibited resistance to infection (Powell Abel *et al.*, 1986; Register and Beachy, 1988; Prins *et al.*, 2008; Tepfer, 2002). However, other viral sequences, such as the RNA-dependent RNA polymerase readthrough domain of TMV, were also shown to induce resistance (Golembowski *et al.*, 1990), as well as the movement of protein (Malyshenko *et al.*, 1993), proteinase (Maiti *et al.*, 1993; Vardi *et al.*, 1993), satellite RNA (Gerlach *et al.*, 1987; Harrison *et al.*, 1987), defective interfering RNA (Kollar *et al.*, 1993), and 5′ (Stanley *et al.*, 1990; Nelson *et al.*, 1987) and 3′ (Zaccomer *et al.*, 1993) non-coding regions. It has become apparent that almost any viral genetic element could be used to confer resistance to virus infection in plants (Gottula and Fuchs, 2009).

The viral transgene protein product is not needed for engineered resistance but there is an inverse correlation between transgene expression and resistance to virus infection (Dougherty *et al.*, 1994). Therefore, the mRNA sequence is responsible for the resistance phenotype rather than the protein itself (Gottula and Fuchs, 2009), and the majority of pathogen-derived resistance phenomena seem to work through RNA-mediated mechanisms (Baulcombe, 2007; Eamens *et al.*, 2008; Prins *et al.*, 2008; Voinnet, 2008). Plant RNA-dependent RNA polymerase and double-stranded RNAase activities were proposed to be part of the mechanism of resistance by producing short RNA of 10−20 nucleotides in length complementary in sequence to the RNA to be degraded from the transgene RNAs (Lindbo *et al.*, 1993). These short RNAs would target specific RNAs for degradation by a dsRNase activity (Dougherty and Parks, 1995). These findings paved the way to the discovery of RNA silencing as a potent defense mechanism against plant viruses (Baulcombe, 2004, 2007; Eamens *et al.*, 2008; Lin *et al.*, 2007; Prins *et al.*, 2008; Voinnet, 2001, 2005, 2008; Waterhouse *et al.*, 1999, 2001).

RNA silencing is initiated by double-stranded RNA (dsRNA) structures that are identical to the RNA to be degraded (Waterhouse *et al.*, 1998). Silencing is associated with the production of 21−25 nucleotide duplexes called small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Hamilton *et al.*, 2002). The siRNAs are produced from

dsRNA precursors by an endonuclease known as Dicer and become incorporated and converted to single-stranded RNAs (ssRNAs) in an Argonaute-containing ribonuclease complex (RISC) that targets RNA for cleavage (Deleris *et al.*, 2006; Hannon, 2002; Obbard *et al.*, 2009; Voinnet, 2001, 2005, 2008). The pioneering work by Baulcombe and Waterhouse and their respective colleagues showed that RNA silencing is an innate and potent plant response to virus infection and a natural example of the concept of pathogen-derived virus resistance (Gottula and Fuchs, 2009).

2. Commercialized Virus-Resistant Transgenic Crops

a. Virus-Resistant Summer Squash

Summer squash expressing the coat protein gene of *Zucchini yellow mosaic virus* and *Watermelon mosaic virus* received exemption status in the USA in 1994 and was released thereafter. This was the first disease-resistant transgenic crop to be commercialized in the USA. Plants of line ZW-20 remain vigorous following exposure to aphid-mediated transmission of *Zucchini yellow mosaic virus* and *watermelon mosaic virus* and produce marketable fruits unlike conventional squash. Summer squash expressing the coat protein gene of *Cucumber mosaic virus*, *Watermelon mosaic virus*, and *Zucchini yellow mosaic virus* was deregulated and commercialized in 1996. Subsequently, numerous squash types and cultivars have been developed by crosses and backcrosses with the two initially deregulated lines. Currently there are five zucchini and six straightneck or crookneck yellow squash cultivars for which combinations of resistance to *Zucchini yellow mosaic virus* and *Watermelon mosaic virus* or resistance to all three viruses are available (Gottula and Fuchs, 2009).

The adoption of virus-resistant summer squash cultivars has increased steadily since 1996. In 2006, the adoption rate was estimated to be 22% (3250 ha) across the USA. The benefit to growers was estimated to be $24 million in 2006 (Gottula and Fuchs, 2009).

b. Virus-Resistant Papaya

Papaya expressing the coat protein gene of *Papaya ringspot virus* was deregulated in 1998 and commercialized in Hawaii. *Papaya ringspot virus* is a major limiting factor to papaya production in Hawaii and around the world. After extensive experimental testing, *Papaya ringspot virus*-resistant papaya was released in 1998 as devastation caused by the virus reached record proportions in the archipelago's main production region (Gonsalves, 1998). The impact of *Papaya ringspot virus*-resistant papaya on the papaya industry in Hawaii is evidenced by its rapid adoption rate. In 2000, the first wave of transgenic papaya bore fruit on more than 42% of the total acreage (Johnson *et al.*, 2007). Resumption of fruitful harvests put papaya packing houses back in business and provided a $4.3 million impact over a 6-year period (Gottula and Fuchs, 2009). By 2006, transgenic papaya cultivars were planted on more than 90% of the total papaya land in Hawaii (780 of a total 866 ha) (Johnson *et al.*, 2007).

Another PRSV-resistant papaya has been deregulated by two of the three US biotechnology regulatory authorities. Line X17-2 differs from the previously deregulated Hawaiian papaya in that it expresses the coat protein gene of a Florida isolate of *Papaya ringspot virus* and is suitable for cultivation in Florida (Davis, 2004). The US Department of Agriculture (USDA) Animal and Public Health Inspection Service (APHIS) and the FDA have granted X17-2 deregulated status (Shea, 2009).

c. Virus-Resistant Potato

Two virus-resistant potato lines were deregulated in 1998 and 2000 in the USA. After failed attempts to create a potato line resistant to *Potato leafroll virus* (PLRV) by coat protein gene expression, lines expressing a PLRV replicase gene were created, field tested, deregulated, and commercialized (Kaniewski and Thomas, 2004). Later, this resistance was stacked with a synthetic *Bt* gene that conferred resistance to Colorado potato beetle. Another potato cultivar was developed by adding the coat protein gene of PLRV. Although many growers in the Pacific Northwest, Midwest USA, and Canada were growing transgenic potato, and no resistance breakage or any detrimental impact on the environmental or human health was reported, virus-resistant potatoes were withdrawn from the market after the 2001 season owing to the reluctance of several large processors and exporters to adopt these products (Kaniewski and Thomas, 2004).

The PLRV-resistant line, called New Leaf Plus Potatoes, has been genetically engineered to express the Cry3a protein from *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) and the *orf1/orf2* gene from PLRV. The mechanism of resistance provided by the *orf1/orf2* gene has not been elucidated at this time. Possible scenarios responsible for the inhibition of viral replication include:

- protein-driven inhibition (viral replication inhibited by the three proteins that could be encoded by the *orf1/orf2* gene)
- RNA-driven inhibition.

Protein-driven inhibition as a mechanism of action of the *orf1/orf2* gene is questionable owing to the lack of conclusive evidence that any of the potential proteins encoded for by the *orf1/orf2* gene are expressed.

d. Virus-Resistant Plum

On 7 May 2010, the Environmental Protection Agency (EPA) registered the pesticide product C5 HoneySweet Plum, which contains the new plant-incorporated protectant (PIP) active ingredient Coat Protein Gene of Plum Pox Virus (CPG-PPV) (EPA 2010 PC Code: 006354).

Plum pox virus (PPV) is a plant virus that reduces the quality of stone fruits, and eventually renders infected trees incapable of producing fruit. It was first described in Europe in 1915, where it was considered to be the most devastating viral disease of stone fruit. PPV is also present in the USA and Canada. Recent outbreaks in New York and Michigan underscore that PPV is becoming endemic despite containment efforts (bulldozing and disposal of infected vegetation, moratoria on the movement/transport of infected plant materials, and control of insect vectors).

PPV is an agricultural pest that causes significant economic losses to the stone fruit industry. The primary effects of the infection are reduction of fruit quality and crop yield. Stone fruits (including native or wild *Prunus* species) that are affected by PPV include plums, peaches, almonds, nectarines, apricots, and sweet and sour cherries. Various other non-*Prunus* dicotyledonous plants have been infected under experimental conditions.

When PPV infects a plant, its genetic material (a single strand of RNA) is inserted into the plant cells. This strand of RNA contains the genes needed to make new virions. One of these genes codes for the PPV coat protein: CPG-PPV. The infected cell makes viral coat protein in a similar manner as it produces its own plant proteins. RNA coding for the CPG-PPV are translated into the amino acid sequences that make up the protein. During virus replication, segments of dsRNA are produced but, ultimately, exact copies of the original single-stranded virus RNA chromosome are formed and packaged together with the coat proteins into new PPV virions. Small segments of dsRNA are formed during this process, and recognized by a defense mechanism within the host plant, post-transcriptional gene silencing. Post-transcriptional gene silencing blocks the transcription as well as the production of viral proteins and RNA. This sequence of events results in the development of natural resistance to further PPV infections, but not before fruit degradation, leaf chlorosis, and other serious damage caused by the virus have occurred (EPA 2010 PC Code: 006354).

Uninfected plum trees can be genetically engineered to express the CPG-PPV. But, because the CPG-PPV is responsible for only one component needed for the production of new virions, these engineered plum trees cannot produce the virus. The USDA Agricultural Research Service, Appalachian Fruit Research Station, has developed a genetically engineered plum tree, called the C5 HoneySweet Plum (C5 or C5 plum), which expresses the CPG-PPV and is resistant to PPV infection. To create the C5 plum, the CPG-PPV is isolated and inserted into the plum genome as a transgene. During the plant's naturally occurring cellular processes, the transgenic CPG-PPV gene is transcribed. The mRNA copied from the inserted viral coat proteins genes forms abnormal regions of dsRNA, and the pathogen-derived virus-resistant mechanism recognizes the abnormality and destroys segments with the same sequence. This process establishes the ability of the plant to respond quickly to a PPV infection, blocking the production of new virions and the spread of the disease (EPA 2010 PC Code: 006354).

The active ingredient of the inserted cassette is a reverse transcription derivation of the virus coat protein RNA, inserted with a $3'$ untranslated region with fusion of a start codon and short leader sequence and an *A. tumefaciens NOS* terminator under direction of a *CaMV 35s* promoter. There is one complete copy of the PPV-CP gene, a small fragment of the PPV-CP *35s* promoter on another insert, and a third insert that is a $3'-3'$ tail-to-tail copy of the PPV-CP with the *35S* promoter for each copy and a portion of GUS sequence flanking each PPV-CP copy. The insert is flanked by plum DNA.

e. Potential Food Safety Issues of Virus-Resistant Transgenic Plants

Since nucleic acids are universal in living organisms, the only novel molecules that are expected to exist in virus-resistant transgenic plants that could have a food safety impact are the proteins synthesized from the transgenes. From a practical perspective, this primarily concerns viral coat proteins, since the vast majority of virus-resistant transgenic plants developed so far, and all but one of the virus-resistant transgenic plants that have been deregulated or authorized for large-scale release, are ones that express coat protein transgenes. It has been argued that there is a long

history of consumption of viral coat proteins in virus-infected non-transgenic plant foods that we consume regularly. Thus, although they may be transgene encoded, coat proteins are in fact very much familiar parts of human and animal diets (Prins *et al.*, 2008) and there is no evidence for adverse health effects of the consumption of the currently deregulated virus-resistant squash and papaya cultivars (Fuchs and Gonsalves, 2007).

Transgenes that express no protein, as is the case for those used in post-transcriptional gene silencing and artificial microRNA approaches, should in theory not pose this sort of question (Fuchs and Gonsalves, 2007).

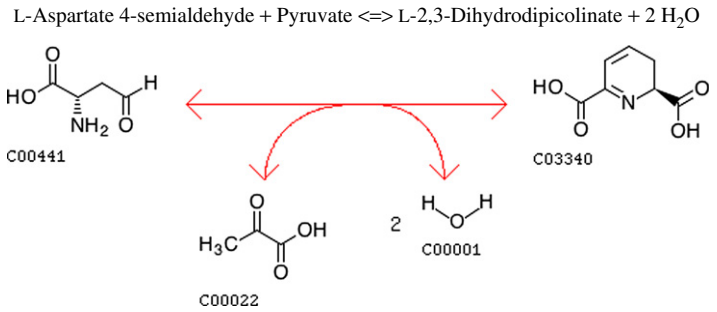
The presence of antibiotic resistance markers in virus-resistant transgenic plants seems to pose no negative health risk. Papaya ringspot virus-resistant transgenic papaya contain two of the selection marker genes for antibiotic resistance, *npt II* and *gus*, and the consumption of millions of kilograms of transgenic papaya fruits over the past years has not provided any evidence of compromising safety. It is postulated that the usefulness of the *npt II* gene far outweighs any risks that might evolve from its presence in transgenic plants (Fuchs and Gonsalves, 2007).

3. Altered Nutrient Profiles in Transgenic Crops

a. High-Lysine Corn

Corn does not contain a significant amount of lysine compared to other dietary sources. Conventional corn–soy-based diets for swine and chicken are characteristically deficient in lysine and require the addition of supplemental lysine for optimal animal growth and performance. Therefore, a genetically modified corn line was developed to have higher levels of lysine in the grain. This corn line is used for feed and food use.

Dihydrodipicolinate synthase is the key enzyme in lysine biosynthesis (Figure 13.7). Dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) cleaves carbon oxygen bonds in the following reaction (KEGG R02292):



The *cordapA* gene from *Corynebacterium glutamicum*, which encodes the bacterial enzyme dihydrodipicolinate synthase, was incorporated into maize. The derived corn line is referred to as corn line LY038 or 'MAVERA HVC with Lysine', where HVC stands for High-Value Corn. The bacterial DHDPS enzyme, unlike the plant DHDPS enzyme, is not sensitive to lysine feedback inhibition, so lysine biosynthesis will continue in the presence of high levels of free lysine (FSANZ A549).

The genetic modification did indeed elevate the lysine levels in the corn; however, four amino acids are significantly reduced in LY038 corn compared to its control, and with the exception of glutamic acid, all are essential amino acids (histidine, isoleucine, and phenylalanine). However, when compared to conventional corn data, the reductions in these amino acids in LY038 corn are shown to remain within the normal variation observed in corn grain.

The high-lysine corn line LY038 is primarily intended for use as field corn for animal feed. Some types of food products that could contain ingredients derived from corn line LY038 in the case of inadvertent co-mingling are: margarine, cooking oil and baking and frying fats; various sweeteners including high fructose, dextrose, and maltodextrins; corn grain used as an additive; flaking grits used almost exclusively in the manufacture of corn flakes; fine grits utilized by the snack, breakfast cereal, and brewing industries; coarse grits eaten as a breakfast food; corn flour; dried-milled corn products used as a substrate for brewing beer; and corn grits and whole kernels used to produce many distilled hard liquors. Owing to the change in lysine levels, there is also the possibility of the occurrence of different Maillard reactions, which can make lysine unavailable by forming complexes.

The *cordapA* gene is under the control of the *Zea mays globulin 1* (*Glb1*) promoter, which in wild-type maize directs expression of the most abundant embryo-specific protein in maize grain. The utilization of the Glb1 promoter for *cordapA* transcription results in the expression of cDHDPS and the accumulation of lysine predominantly in the germ portion of the grain. Following the promoter is an intron sequence derived from the rice *actin-1* gene, the

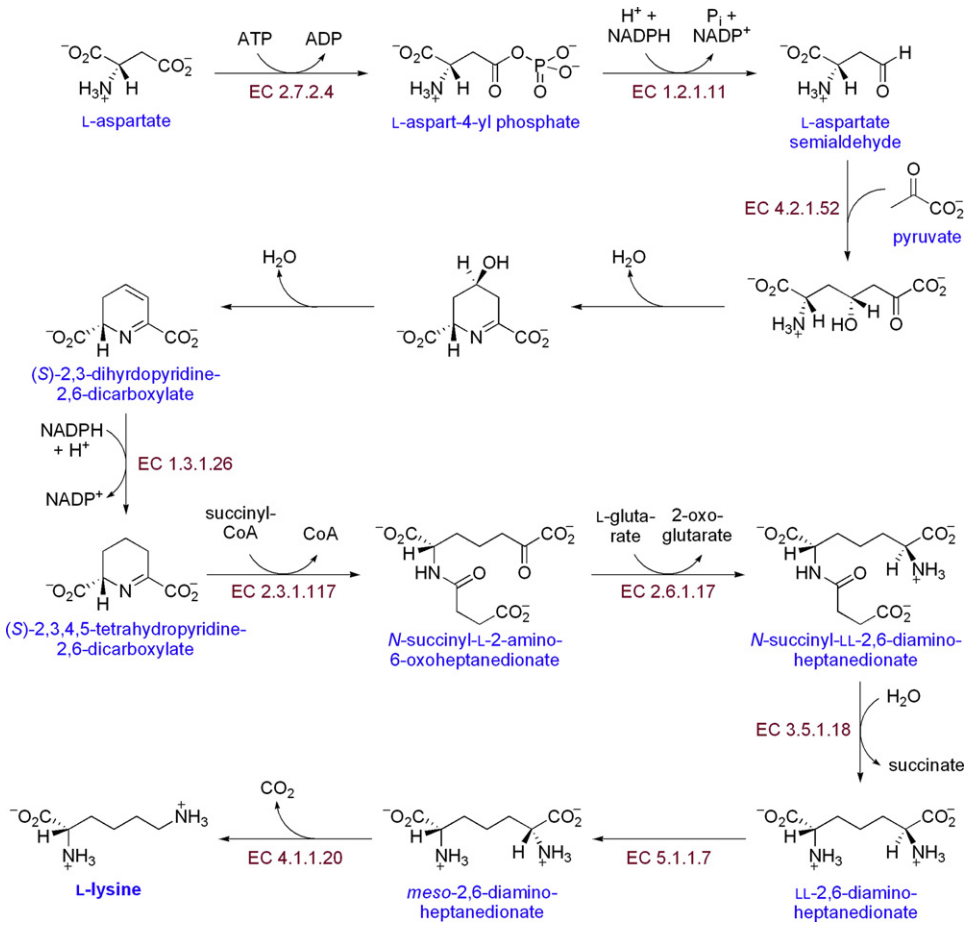


FIGURE 13.7 The lysine biosynthesis pathway. In corn, a key enzyme of the pathway, dihydrodipicolinate synthase (EC 4.2.1.52) is inhibited by lysine feedback. When an additional nonsensitive dihydrodipicolinate synthase from *Corynebacterium glutamicum* is expressed in corn, the lysine content is elevated *(Source: http://en.wikipedia.org/wiki/File:Lysine_Biosynthesis.png).*

FIGURE 13.8 Organization of the T-DNA cassette for the *cordapA* gene from *Corynebacterium glutamicum*, which encodes the bacterial enzyme dihydrodipicolinate synthase. This cassette is integrated into the genome of maize to enhance lysine biosynthesis. Glb1 promoter: *Zea mays* globulin 1 promoter; Act1 intron: rice actin-1 intron; DHDPS CTP: *Zea mays* dihydrodipicolinate synthase chloroplast transit peptide directing the gene product to the plastid; *cordapA*: gene encoding *Corynebacterium glutamicum* dihydrodipicolinate synthase; *Glb1* 3'UTR: *Zea mays* globulin 1 3' untranslated region to terminate transcription.

rAct1 intron, which enhances DNA transcription. The *cordapA* gene is preceded by the *Zea mays* dihydrodipicolinate synthase chloroplast transit peptide (mDHDPS CTP), to translocate cDHDPS to the plastid, where the majority of amino acid biosynthesis occurs. The 3′ non-translated region of the globulin 1 gene follows the *cordapA* gene and contains the polyadenylation signal that directs the termination and maturation of the *cordapA* transcript (FSANZ A549) (Figure 13.8).

III. GENETICALLY MODIFIED MICROORGANISMS AND DERIVED PRODUCTS INTENDED FOR FOOD USE

Genetically modified microorganisms (GMMs) are found in food, involved in the production of food or additives. These microorganisms can be archaea, bacteria, and eukarya. Eukarya include filamentous fungi, yeasts, protozoa,

and microalgae. Owing to the ease of genetic manipulation in microorganisms a high diversity of GMMs and their products exists in food. The European Food Safety Authority (EFSA, 2011) defines four categories:

- Category 1: Chemically defined purified compounds and their mixtures in which both GMMs and newly introduced genes have been removed (e.g. amino acids, vitamins).
- Category 2: Complex products in which both GMMs and newly introduced genes are no longer present (e.g. cell extracts, most enzyme preparations).
- Category 3: Products derived from GMMs in which GMMs capable of multiplication or of transferring genes are not present, but in which newly introduced genes are still present (e.g. heat-inactivated starter cultures).
- Category 4: Products consisting of or containing GMMs capable of multiplication or of transferring genes (e.g. live starter cultures for fermented foods and feed).

The different categories demonstrate the complex nature of products made with recombinant technology, ranging from a single isolated compound produced by a GMM to pure cultures of viable GMMs. Amino acids or vitamins that have been purified by crystallization would represent examples at one end of this spectrum and microbial food cultures such as probiotics or dairy starters at the other. Since GMMs are the 'workhorses' in molecular biology, an enormous body of information is available on heterologous expression systems with a potential application in food, which it is not possible to address here. Therefore, this section will focus on viable GMMs in food and the major protein products derived from heterologous systems, which are currently generally recognized as safe by major regulatory bodies, such as the US FDA, the EFSA and Food Standards Australia New Zealand (FSANZ) (Table 13.4). It is assumed that if a product is approved it is applied to foods.

A. Products Consisting of or Containing Genetically Modified Microorganisms Capable of Multiplication

1. Modified Yeast to Reduce Hydrogen Sulfide in Fermented Foods and Beverages

The production of volatile sulfur compounds such as hydrogen sulfide (H_2S) during alcoholic fermentation is a problem that affects the brewing, wine-making, and sake industries. Hydrogen sulfide is an undesirable by-product of the sulfate assimilation pathway formed in *Saccharomyces cerevisiae* during fermentation (Figure 13.9).

The undesirable characteristics of H_2S include introducing a rotten egg odor to the fermented beverage, which can render the product unsaleable. It also has the potential to form mercaptans and thiols which not only are dangerously reactive compounds, but also impart onion and canned vegetable aromas. The removal of these undesirable sulfur compounds is technically difficult and strips the wine of desirable flavor compounds. These sensory characteristics are extremely important to the wine producer. The current method used in the wine industry to remove sulfides from the wine is to add copper in attempts to chelate the sulfur. Copper can lead to the catalysis of deleterious compositional changes and increase the amount of waste produced by wineries. The use of copper as a fining agent may also lead to high residual copper levels in wine, which is allowed up to 0.5 mg/l for wine (FDA-GRN 350).

The commercial *S. cerevisiae* strain P1Y0, which does not produce H_2S as a by-product of fermentation, was developed by replacement of its native *MET10* gene with the 'low-H_2S' *MET10* allele from the *S. cerevisiae* wine yeast UCD932. The *MET10* gene codes for subunit α of assimilatory sulfite reductase (EC 1.8.1.2), which catalyzes the six-electron reduction of sulfite into sulfide (KEGG R00858):

Hydrogen sulfide + 3 NADP+ + 3 H_2O <=> Sulfite + 3 NADPH + 3 H^+

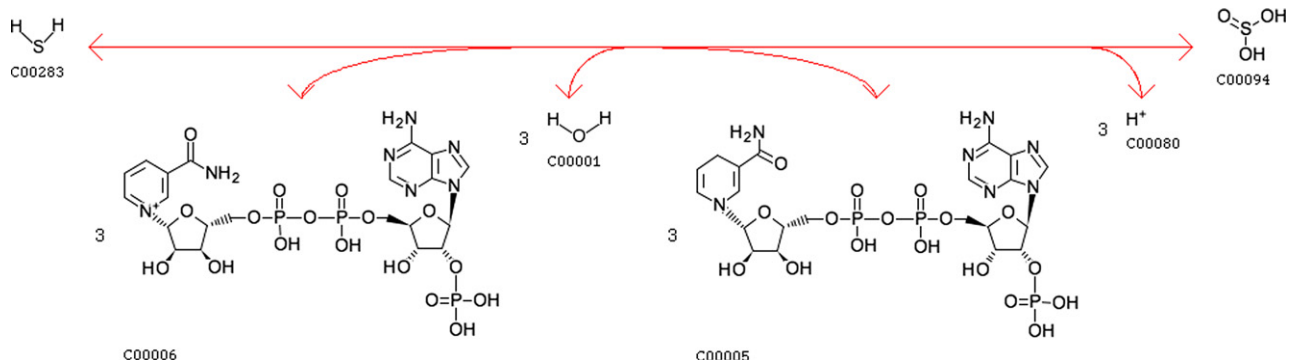


TABLE 13.4 Recombinant Microorganisms and Enzymes Regulated by Major International Agencies such as the US Food and Drug Administration (FDA), European Food Safety Authority (EFSA), and Food Standards Australia New Zealand (FSANZ)

Source Microorganism	Enzyme	Reference and Trade name
Aspergillus niger	Asparaginase/*Aspergillus niger*	FDA-GRN 214, FSANZ A1003, PreventASe™
	Carboxypeptidase/*Aspergillus niger*	FDA-GRN 345, Accelerzyme® CPG
	Chymosin B/*Bos taurus*	FDA-GRN 230, 21 CFR 184.1685
	Lipase (triacylglycerol)/synthetic construct of various *Fusarium* species	FDA-GRN 296, Panamore™
	Lipase (triacylglycerol)/*Candida antarctica*	FDA-GRN 158, Lipozyme®
	Phospholipase A_2/*Sus scrofa* (porcine)	FDA-GRN 183, Bakezyme, Cakezyme, Maxapal A2
Aspergillus oryzae	Asparaginase/*Aspergillus oryzae*	FDA-GRN 201, Acrylaway®
	Aspartic proteinase/*Rhizomucor miehei*	FDA-GRN 34, NovoCarne™ Tender
	Glucose oxidase/*Aspergillus niger*	FDA-GRN 106, Gluzyme®
	Laccase/*Myceliophthora themophila*	FDA-GRN 122
	Lipase (triacylglycerol)/*Thermomyces lanuginosus*	FDA-GRN 43
	Lipase (triacylglycerol)/*Fusarium oxysporum*	FDA-GRN 75
	Lipase (triacylglycerol)/hybrid lipase from *Thermomyces lanuginosud/fusarium oxysporum*	FDA-GRN 103
	Lipase (triacylglycerol)/*Rhizomucor miehei*	FSANZ A402, palatase
	Pectin esterase/*Aspergillus aculeatus*	FDA-GRN 8
	Phospholipase A_1/*Fusarium venenatum*	FDA-GRN 142, Novozyme® 46016
	Phytase/*Peniophora lycii*	FSANZ A371
	Xylanase/*Thermomyces lanuginosus*	FDA-GRN 54
Bacillus licheniformis	α-Amylase/*Bacillus stearothemophilus*	FDA-GRN 24
	α-Amylase/*Bacillus licheniformis* + *Bacillus amyloliquefaciens* modified gene	
	Termamyl® LC	FDA-GRN 22
	α-Amylase/*Bacillus licheniformis modified gene*	FDA-GRN 79, Novozym® 28035
	Exo-maltotetraohydrolase, G4 amylase/*Pseudomonas saccharophila stutzeri*	FDA-GRN 277
	Glycerophospholipid cholesterol acyltransferase/*Aeromonas salmonicida*	FDA-GRN 265, FoodPro™, LysoMaxa Oil
	Pullulanase/*Bacillus deramificans*	FDA-GRN 72
Bacillus subtilis	α-Acetolactate decarboxylase/*Bacillus brevis*	21 CFR 173.115
	Maltogenic amylase/*Bacillus stearothermophilus*	GRASP 7G0326
	Pullulanase/*Bacillus naganoensis*	FDA-GRN 20, GRASP 4G0293
	Pullulanase/*Bacillus acidopullulyticus*	FDA-GRN 205
	Branching glycosyltransferase/*Rhodothermus obamensis*	FDA-GRN 274

TABLE 13.4 Recombinant Microorganisms and Enzymes Regulated by Major International Agencies such as the US Food and Drug Administration (FDA), European Food Safety Authority (EFSA), and Food Standards Australia New Zealand (FSANZ)—cont'd

Source Microorganism	Enzyme	Reference and Trade name
Escherichia coli K-12	Chymosin	21 CFR 184.1685
	Cyclodextrin glucanotransferase/*Klebsiella oxytoca*	FDA-GRN 155, FDA-GRN 64
Fusarium venenatum	Xylanase/*Thermomyces lanuginosus*	FDA-GRN 54
Hansenula polymorpha yeast	Hexose oxidase/*Chondrus crispus*	FSANZ A475, FDA-GRN 238
	Lipase (triacylglycerol)/*Fusarium heterosuorum*	FDA-GRN 238, FSANZ A569, GRINDAMYL™ POWERBake
Kluyveromyces marxianus var. *lactis*	Chymosin B/*Bos taurus*	21 CFR 184.1685
Myceliophthora thermophila	Cellulase/*Myceliophthora thermophila*	FDA-GRN 292
Pichia pastoris	Phospholipase C/DNA fragment from undetermined soil organism	FDA-GRN 204
Pseudomonas fluorescens Biovar I	α-Amylase/*Thermococcales hybrid modified gene*	FDA-GRN 126
Saccharomyces cerevisiae	Ice structuring protein/*Macrozoarces americanus* (ocean pout)	FDA-GRN 117
	Urea amidolyase/*Saccharomyces cerevisiae*	FDA-GRN 175
	Subunit α of assimilatory sulfite reductase, 'low-H_2S' *MET10*/*Saccharomyces cerevisiae*	FDA-GRN 350
	Malolactic enzyme/*Oenococcus oeni*	
	+ Malate permease/*Schizosaccharomyces pombe*	FDA-GRN 120
Streptomyces violaceruber	Phospholipase A_2/*Streptomyces violaceruber*	FDA-GRN 212, PLA2 Nagase
Trichoderma reesei	Aspergillopepsin I/*Trichoderma reesei*	FDA-GRN 333
	Chymosin B/*Bos taurus*	FDA-GRN 230, Chymostar Supreme
	Glucoamylase/*Trichoderma reesei*	FDA-GRN 372
	Pectin lyase/*Aspergillus niger*	FDA-GRN 32
	Transglucosidase/*Aspergillus niger*	FDA-GRN 315

The low-H_2S *MET10* allele differs by three single-nucleotide polymorphisms from the wild-type allele (C404A, G1278A, and C1985A). The single-nucleotide polymorphisms found at positions 404 and 1278 are common to a number of strains and the one found at position 1985 is unique to the *MET10* coding sequence of UCD932 (and now the *MET10* coding sequence of P1YO). Only two of these polymorphisms result in amino acid changes since the change at nucleotide position 1278 is a silent mutation. The two copies of the wild-type *MET10* gene were completely removed from the genome of the final low-H_2S commercial strain by knocking out the genes with markers *kanMX* and *hphMX*, via homologous recombination with the non-coding upstream and downstream *MET10* flanking sequences. Subsequently, the markers were replaced with the low-H_2S *MET10* allele of UCD932. The low-H_2S *MET10* allele is under the control of its intrinsic 5′ and 3′ flanking sequences, as seen in its expression cassette (Figure 13.10) (FDA-GRN 350).

The *MET10* allele swap results in minimal H_2S leakage from the yeast cell. The P1Y0 active dry yeast is used as a starter culture for alcoholic beverage fermentation such as grape must, brewing wort, and rice fermentations, and is applied to wine, champagne, sherry, sake, beer, brandy, cognac, whiskey, and rum.

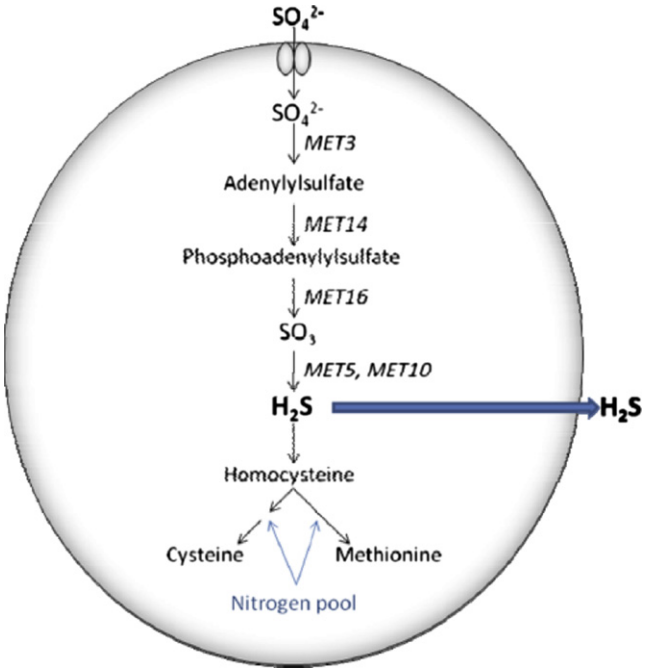
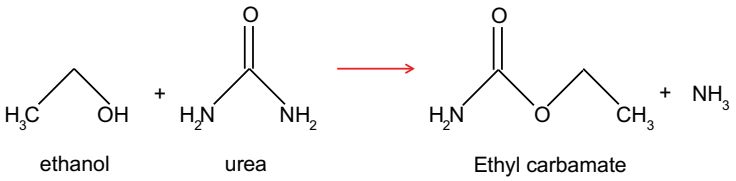


FIGURE 13.9 Pathway of the production of hydrogen sulfite during fermentation. The MET10 enzyme catalyzes the last step in hydrogen sulfite production. The replacement of the native enzyme with a 'low-H2S' transgenic isoform results in low sulfite production. *(Sweiegers and Pretorius, 2007)*

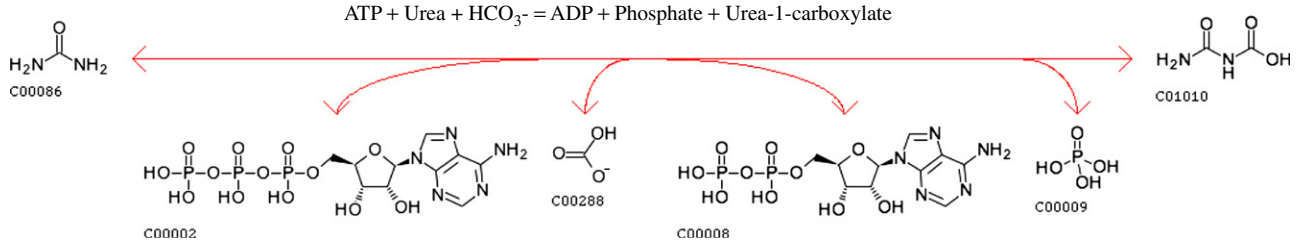
2. Modified Yeast for Reduction of Ethyl Carbamate in Fermented Beverages

Certain fermented foods and alcoholic beverages contain ethyl carbamate (Urethane, CAS No. 5-179-6) (see Zimmerli and Schlatter, 1991, for review), which is a known carcinogen in animals and a suspected carcinogen in humans (12th Report on Carcinogens 2011 by the US National Toxicology Program). Ethyl carbamate forms in a reaction of ethanol with cyanate or urea. In *S. cerevisiae* urea is formed as a breakdown product of L-arginine, and the reaction of ethanol and urea is known to be the primary source of ethyl carbamate in grape-based and rice-based fermented products (FDA-GRN 175):

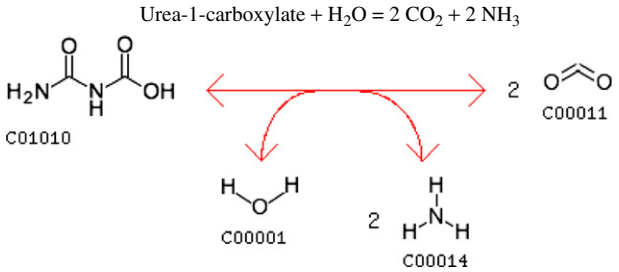


Urea is normally metabolized in *S. cerevisiae* by the enzyme urea amidolyase, exhibiting urea carboxylase (EC 6.3.4.6) as well as allophanate hydrolase (EC 3.5.1.54) activity:

Urea carboxylase activity (EC 6.3.4.6, KEGG R00774):



Allophanate hydrolase activity (EC 3.5.1.54, KEGG R00005):



Urea amidolyase is encoded by the *S. cerevisiae DUR1,2* gene (systematic name YBR208C). During fermentation, in the presence of good nitrogen sources the *DUR1,2* gene is only weakly expressed and therefore urea is poorly metabolized. As a result, urea accumulates in the yeast cell and releases into the wine, where it reacts with ethanol to form ethyl carbamate. Heating and aging of the wine accelerate the production of ethyl carbamate, and therefore the levels are much higher in sherry and sake/rice wine (Zimmerli and Schlatter, 1991, FDA-GRN 175).

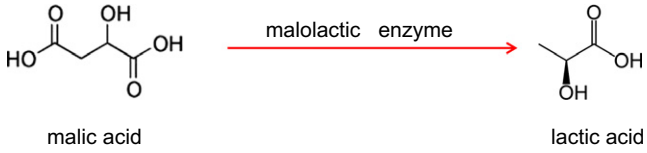
To reduce the formation of ethyl carbamate the genetically modified *S. cerevisiae* strain ECMoOl was developed, where a *DUR1,2* containing expression cassette is integrated into the *Ura3* locus by homologous recombination of flanking sequences (*URA3* 5′- and *URA3* 3′-flanks). The *DUR1,2* gene expression is controlled by the *S. cerevisiae PGK1* promoter (*PGK1*p) and terminator (*PGK1*t) sequences (FDA-GRN 175):

As a result, the expression of the *DUR1,2* gene is increased by 17-fold in the recombinant strain ECMoOl, causing a more effective degradation of urea during alcoholic fermentation, resulting in 89% reduced ethyl carbamate levels (Figure 13.11).

The ECMoOl active dry yeast is used as a yeast starter culture for alcoholic beverage fermentation such as grape must and rice fermentations (FDA-GRN 175).

3. Modified Yeast for Malolactic Fermentation

Alcoholic fermentation and malolactic fermentation are the two main biotechnological processes in wine making. After the alcoholic fermentation, most red wines and some white wines are subjected to malolactic fermentation. This secondary fermentation, usually carried out by lactic acid bacteria, is important for deacidification, flavor modification, and microbial stability of the wine. Wine deacidification takes place through the conversion of L-malate (dicarboxylic acid) to L-lactate (monocarboxylic acid). *Oenococcus oeni* is the major lactic acid bacterium responsible for malolactic fermentation of wines, but most wine lactic acid bacteria possess the malolactic enzyme which catalyzes the L-malate to L-lactate conversion:



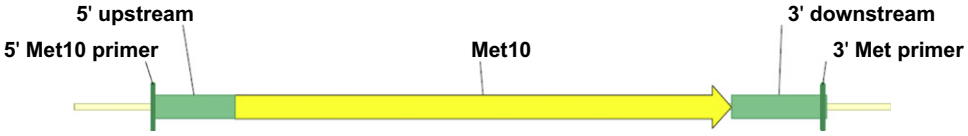


FIGURE 13.10 Expression cassette for the 'low-H2S' *MET10* allele. The 'low-H2S' *MET10* gene obtained from a different yeast strain is flanked by intrinsic 5' upstream and 3' downstream sequences that were used for homologous recombination into the *S. cerevisiae* wild-type *MET10* gene locus.

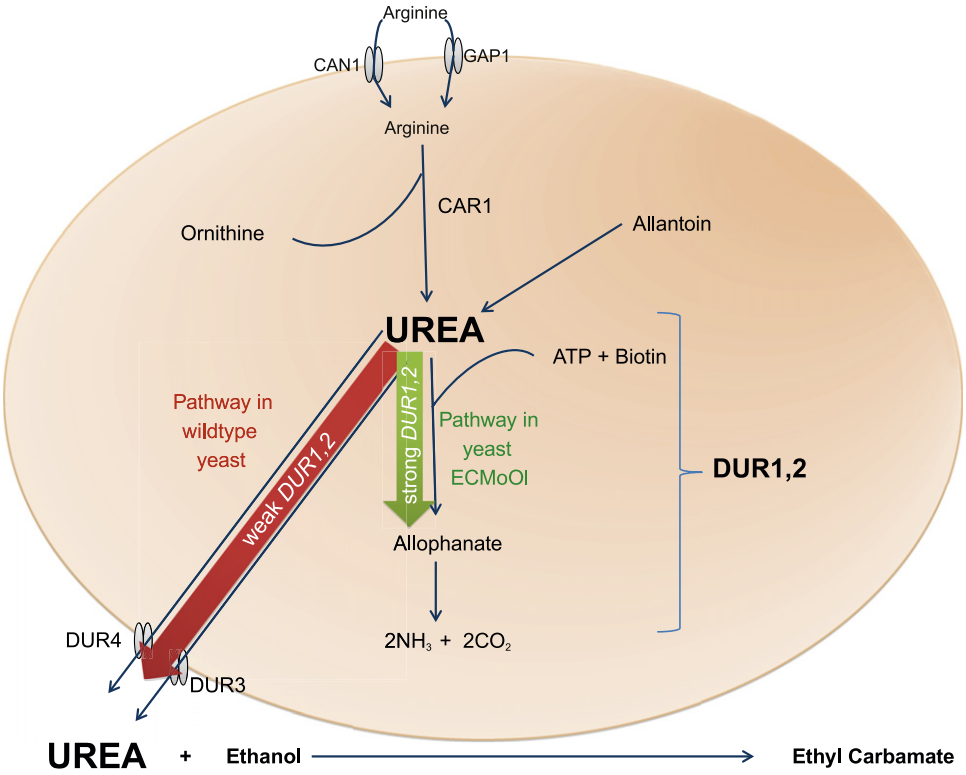


FIGURE 13.11 Degradation of urea by DUR1,2 urea amidolase. High DUR1,2 activity degrades urea effectively. However, in high nitrogen conditions activity is low resulting in urea accumulation in the wine and detrimental downstream reactions. The recombinant *ECMoOl* yeast with constitutive high DUR1,2 expression was developed to prevent urea excretion. *(Coulon et al., 2006)*

In contrast to the desired effects of malolactic fermentation, some lactic acid bacteria strains also show specific metabolic pathways which can lead to the formation of undesirable compounds. In addition, due to the sensitivity of lactic acid bacteria to nutritional conditions, pH, temperature, sulfur dioxide content, and ethanol concentration of the wine, malolactic fermentation is often unpredictable and difficult to achieve, even with the use of commercial starter cultures of lactic acid bacteria (FDA-GRN 120).

Saccharomyces cerevisiae is unable to efficiently degrade L-malate, but the yeast *Schizosaccharormyces pombe* is able to do so; however, it is unsuitable for the production of wine. Therefore, the genetically enhanced strain ML01 of *S. cerevisiae* was created to aid malolactic fermentation. To overcome the lack of malolactic fermentation, as well as transport of L-malate into the *S. cerevisiae* cell, two genes were transferred into the ML01 strain: the *mleA* gene encoding the malolactic enzyme from *O. oeni* and the *mae1* gene encoding malate permease from *S. pombe* (FDA-GRN 120) (Husnik *et al.*, 2007).

A linear expression cassette containing the tandem *mae1* and the *mleA* genes under the control of the *S. cerevisiae PGK1* promoter (*PGK1*p) and terminator (*PGK1*t) was integrated into the *URA3* (recombination with flanking ura3 sequences) locus of *S. cerevisiae* strain S92 (Husnik *et al.*, 2007) (Figure 13.12).

The resulting ML01 strain is capable of decarboxylating up to 9.2 g/l of malate to equimolar amounts of lactate in Chardonnay grape must during the alcoholic fermentation. The presence of the malolactic cassette in the genome does not affect growth, ethanol production, fermentation kinetics, or metabolism of ML01, is substantially equivalent to the parental industrial wine yeast (Husnik *et al.*, 2007), and is therefore generally regarded as safe by the FDA (FDA-GRN 120).

FIGURE 13.12 The expression cassette containing the *mae1* gene from *S pombe* (encoding malate permease) and the *mleA* gene from *O oeni* (encoding the malolactic enzyme). Expression is controlled by the *S. cerevisiae PGK1* promoter (*PGK1p*) and terminator (*PGK1t*). The ura3 flanking sequence was used for homologous recombination to create the ML0l yeast capable of malolactic fermentation.

The ML01 wine active dry yeast is used as a yeast starter culture for grape must fermentation to perform alcoholic fermentation. However, in contrast to traditional yeast strains, alcoholic and malolactic fermentation occur simultaneously and solely in the yeast. The use of ML01 wine yeast depends on the wine maker's choice and relies on whether the malolactic fermentation is required for deacidification, flavor modification, or microbial stability, or a combination of these characteristics.

If deacidification is the major contribution required from malolactic fermentation, the use of the ML0l wine active dry yeast is highly recommended. The bioconversion of a dicarboxylic acid (L-malate) into a monocarboxylic acid (L-lactate) decreases the total acidity of the wine and softens its mouth-feel. In some wines, such as red wines with aging potential, malolactic fermentation by bacteria contributes not only to deacidification, but also to flavor modification through bacterial secondary metabolism. In this case, it is often preferable not to use the ML0l yeast and to carry out malolactic fermentation with lactic acid bacteria. In contrast, the ML0l yeast is applicable when wine microbial stability is required without the development of buttery flavors (acetoyn, diacetyl) due to lactic acid bacteria secondary metabolism, as is often the case in white wines. The ML0l wine yeast strain can also be used in cases where wine stability is required as soon as possible, since wine microbial stability is enhanced once the malolactic fermentation is completed by removal of L-malate. Moreover, the sooner the malolactic fermentation is completed, the sooner the wine can be sulfited and bottled with a low risk of bacterial contamination (FDA-GRN 120).

IV. COMMERCIAL ENZYMES AND PROTEINS IN THE FOOD INDUSTRY DERIVED FROM GENETICALLY MODIFIED MICROORGANISMS

Enzymes are commonly used in food processing and in the production of food ingredients. Intrinsic enzymes are traditionally isolated from culturable microorganisms, plants, and mammalian tissues. Therefore, their production and isolation can be challenging and the available resources might be limiting. Moreover, enzymes often originate from microorganisms that cannot be easily cultured under industrial conditions or have toxic by-products. By judicious selection of host microorganisms, recombinant production strains can be constructed to allow efficient production of enzymes that are substantially free of undesirable by-products or other microbial metabolites. An ever-increasing number of enzymes is produced using heterologous expression systems, where transgenic technology is used to engineer and introduce transgenes into microbial host strains, which can themselves be genetically modified to optimize the biotechnological process. For example, several microbial strains recently developed for enzyme production have been engineered to increase enzyme yield by deleting native genes encoding extracellular proteases. Moreover, certain fungal production strains have been modified to reduce or eliminate their potential for production of toxic secondary metabolites (Olempska-Beer *et al.*, 2006).

Native enzymes are often not well adapted to the conditions used in modern food production. The increasing sophistication of food processing creates a demand for a broad variety of food-processing enzymes with characteristics compatible with food-processing conditions. Therefore, enzymes are specifically engineered for improved properties in industrial processes through DNA changes which result in an altered amino acid sequence. For example, commonly used sweeteners such as glucose or fructose syrups are typically produced from corn starch using hydrolytic enzymes. In the first step of starch hydrolysis, starch is liquefied with α-amylase by heating at 105°C for 2−5 minutes followed by 1−2 hours at 90−100°C. Thus, α-amylases with increased heat stability and improved compatibility with other parameters of the liquefaction process have been engineered (Olempska-Beer *et al.*, 2006).

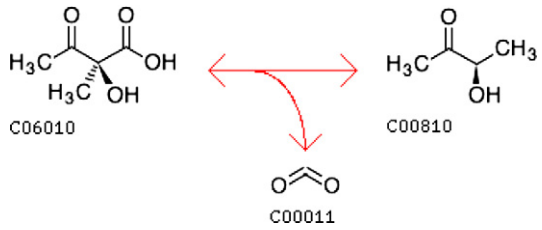
The following paragraphs will focus on the biochemical properties as well as the genetic origin and modifications of recombinant enzymes produced by GMMs which are currently used in the production of food (Table 13.4). We will not discuss safety-related characteristics. The safety evaluation of food processing enzymes from recombinant microorganisms has been extensively discussed in the literature (Jonas *et al.*, 1996; Pariza and Johnson, 2001) and in guidance documents issued by regulatory authorities and international organizations, for example, by the Scientific Committee for Food (SCF, 1992) or the EFSA guidance document on the risk assessment of products derived from GMMs (EFSA, 2011). In principle, the same safety considerations apply to enzymes derived from native and recombinant microorganisms. The key component in evaluating enzyme safety is the safety assessment of the production strain, in particular, its pathogenic and toxigenic potential (Pariza and Johnson, 2001). Although neither pathogenic nor toxigenic microorganisms are intentionally used in the production of food-processing enzymes, certain fungi traditionally used as sources of enzymes have been found to produce low levels of toxic secondary

metabolites under fermentation conditions conducive to the synthesis of these compounds. Some of these microorganisms are now used as sources of recombinant enzymes (Olempska-Beer *et al.*, 2006).

A. Acetolactate Decarboxylase

Acetolactate decarboxylase (EC 4.1.1.5) reduces the undesirable butter aroma occurring as a by-product of beer brewing by converting diacetyl (acetolactate) into the neutral-tasting acetoin (KEGG R02948):

(*S*)-2-Hydroxy-2-methyl-3-oxobutanoate $\rightleftharpoons$ (*R*)-2-Acetoin + CO_2



During storage of beer, the diacetyl aroma is slowly converted into a neutral taste note. In order to shorten the conversion time, diacetyl is eliminated by addition of acetolactate decarboxylase.

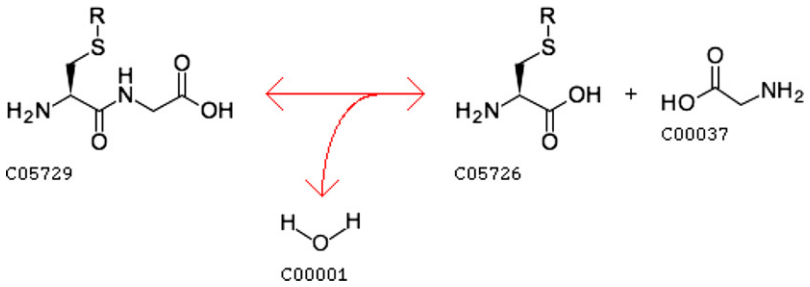
Transgenic acetolactate-decarboxylase is currently derived from a modified *Bacillus subtilis* strain that contains the unmodified gene coding for the *Bacillus brevis* α-acetolactate decarboxylase (21 CFR 173.115).

To date, two biotechnologically produced acetolactate-decarboxylase specimens are marketed in Europe. They are produced with the help of GM brewer's yeast, *S. cerevisae*. In the USA an enzyme preparation from a modified *B. subtilis* strain that contains the gene coding for α-acetolactate decarboxylase from *B. brevis* is permitted in food for human consumption (21 CFR 173.115).

B. Aminopeptidase

Aminopeptidases (EC 3.4.11) are enzymes produced and secreted by glands of the small intestine where they participate in the digestion of proteins, but are widely distributed throughout the animal kingdom and also found in all microorganisms (KEGG database). An aminopeptidase cleaves single amino acids from the N-terminus of protein or peptide substrates. For example, aminopeptidase EC 3.4.11.1 can mediate the cleavage of an N-terminal glycine (KEGG R04951):

R-S-Cysteinylglycine + H_2O <=> S-Substituted L-cysteine + Glycine



These cleavages will change the flavors of the proteins in a food matrix. Therefore, aminopeptidases are used in the production of cheese, beverages, flavorings, meats, and milk products. They are typically meant to enhance and optimize aroma and flavor. The addition of aminopeptidases can accelerate the maturing of cheese. They are also used in animal feeds to improve the utilization of proteins in feed for piglets and poultry. The cleavage of an N-terminal glycine is described here to give an example of the mechanism of aminopeptidases.

Various aminopeptidases have been produced biotechnically for some time utilizing various fungal and bacterial cultures (such as *Aspergillus*, *Lactococcus*, and *Trichoderma*). Among the recombinant peptidases produced are the

aminopeptidase (PepN) from *Lactobacillus rhamnosus* S93, *Lactococcus lactis* (FDA 21CFR184.1985), and *Aspergillus sojae* (European Patent 0967286).

C. Amylases

Amylases are amylolytic enzymes that break down starches into sugars and are very widespread throughout the animal, plant, and microorganism kingdoms (OrthoDB EC 3.2.1). Various amylases exist which cleave the branches of starch molecules with a particular specificity. Depending on the type of amylase, the resulting compounds may be simple sugars such as glucose or fructose, compound sugars such as maltose, malt sugar, or special forms of starch such as dextrins. All amylases are glycoside hydrolases which act upon the α-(1,4)-and/or α-(1,6)-linkages of starch polymers (Goesaert *et al.*, 2009). Figure 13.13 presents an overview of the modes of action of amylolytic enzymes.

Amylases are predominantly used to modify raw materials containing starch in the food-processing industry. The most important area of application of amylases is the production of sugars from starch (glucose syrup, fructose syrup), which later become ingredients in a wide variety of food products, e.g. sweets, baked goods, ice cream, or tomato ketchup. Amylases are naturally present in many raw materials, such as in cereals or yeasts. However, these

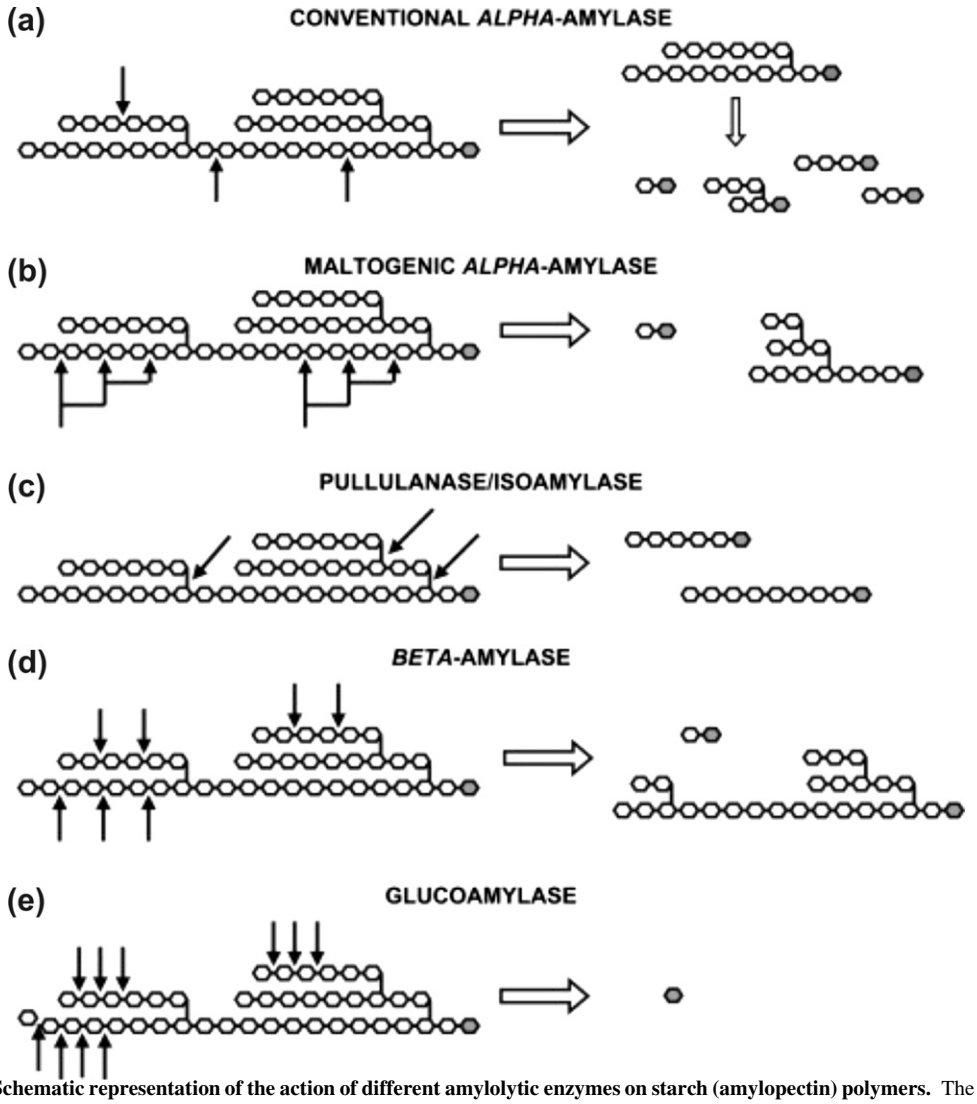


FIGURE 13.13 Schematic representation of the action of different amylolytic enzymes on starch (amylopectin) polymers. The gray ring structure represents a reducing glucose residue. (a) Endo-type action of α-amylase, yielding branched and linear low-molecular-weight dextrins; (b) mainly exo-type action of maltogenic α-amylase, yielding mainly maltose; (c) debranching enzyme action, yielding linear dextrins; (d) purely exo-type action of β-amylase, yielding maltose and β-limit dextrins; (e) purely exo-type action of glucoamylase, yielding glucose. *(From Goesaert* et al.*, 2009.)*

naturally occurring amylases are often either insufficient or too slow in effect. In order to steer or accelerate the degradation of starch, industrially produced amylases are commonly added. These preparations mostly contain a mixture of several types of amylase (GMO Compass).

Amylases are routinely used in baking and bread making for flour standardization and as anti-staling agents. In sound wheat flour, α-amylases are virtually absent, while β-amylases are abundantly present, but the latter have little if any activity on undamaged, native starch granules and are inactivated before starch gelatinization. Therefore, amylase activity in wheat flour is often optimized by adding fungal α-amylases. The added amylases increase the level of fermentable and reducing sugars in flour and dough, thus promoting yeast fermentation and the formation of Maillard reaction products, which, in their turn, intensify bread flavor and crust color. However, amylase functionality may also be related to the reduction of dough viscosity during starch gelatinization, thus prolonging oven rise and resulting in an increased loaf volume. Certain amylases can delay crumb firming and hence function as anti-staling agents. Possible mechanisms or modes of action whereby these enzymes retard the staling/firming process are discussed below. Typical amylase-containing anti-staling products mainly consist of bacterial or fungal α-amylases with intermediate thermostability (Goesaert *et al.*, 2009).

During the production of fermented alcoholic drinks, the starch in the raw materials needs to be broken down into small sugar molecules, to allow the yeast to transform it into alcohol. Enzymes carry out this process in two stages: liquefaction and saccharification. Traditionally, enzymes have been provided by adding malt, which is now being increasingly replaced in distilling operations by the use of isolated enzyme preparations. A comparatively small quantity of industrial enzymes can replace significant amounts of malt.

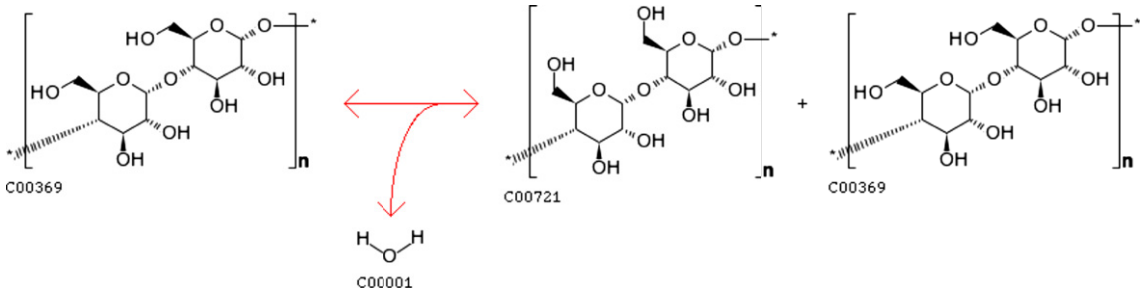
In fruit juices, amylases eliminate lees that contain starch. This is especially important if the fruits are picked while unripe and stored for relatively long periods at low temperature. Under these conditions fruit pulp contains starch in sufficient amounts to cause turbidity or even gelatinize during processing, which makes productive procedures difficult. The addition of amylolytic enzymes counteracts these processes. As feed additives, amylases increase the breakdown of starches and thereby contribute to the better use of plant-based feed.

It has been possible for a long time to produce amylases with a variety of non-genetically modified fungal and bacterial cultures. As a rule, bacterial amylases are more stable in regard to temperature than are amylases derived from fungal cultures. Bacterial amylases now are produced predominantly with GMMs (various types of *Bacillus*). In the production of amylases using fungi, cultures are most often used that are not regarded as genetically modified (GMO Compass).

1. α-Amylases

α-Amylases (EC 3.2.1.1) are enzymes that catalyze the hydrolysis of internal α-1,4-glycosidic linkages in polysaccharides into low-molecular-weight products, such as glucose, maltose, and maltotriose units (de Souza and Magalhaes, 2010). α-Amylases act on starch, glycogen, and related polysaccharides or oligosaccharides in a random manner; reducing groups are liberated in the α-configuration (KEGG R02108):

Starch + H_2O <=> Dextrin + Starch



The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolyzed. A variety of recombinant α-amylase mutants engineered for specific applications are currently produced in *Bacillus subtilis*, *Bacillus licheniformis*, and *Pseudomonas fluorescens* (Olempska-Beer *et al.*, 2006). The *B. licheniformis* α-amylase (*amyl*) gene is commonly used as a transgene and template for modifications (FDA-GRN 79). In addition, various thermostable α-amylases have been isolated from thermophilic

microorganisms and expressed in heterologous production strains. Treatment of starch with α-amylase may be followed by treatment with other suitable enzymes such as glucoamylase, pullulanase, and glucose isomerase.

Termamyl® SC is a preparation of heat-stable α-amylase from *Bacillus stearothemophilus* produced in *B. licheniformis* (GRASP 0G0363, FDA-GRN 24). The sequence of the *B. stearothemophilus* α-amylase *amyS* gene has been modified to increase enzyme stability at low calcium concentrations and low pH. The modified enzyme has a deletion of two amino acids (positions 181 and 182) and a single substitution (position 193) compared to the original *B. stearothemophilus amyS* gene. *Termamyl® SC* is applied in the starch industry for the continuous liquefaction at temperatures of up to 105−110°C, in the alcohol industry for thinning starch in distilling mashes, in brewing to liquefy starch added to wort, and in the sugar industry to break down the starch in cane juice (FDA-GRASP 0G0363, FDA-GRN 24).

Termamyl® LC is a preparation of heat-stable α-amylase from *B. licheniformis* produced in *B. licheniformis* (FDA-GRN 22). The sequence of the *B. licheniformis* α-amylase *amyL* gene has been modified to enable operation at lower pH and lower calcium levels than conventional thermostable α-amylases. The template for the *Termamyl® LC* enzyme was the *B. lichenifomis* α-amylase *amyS* gene used in the production of *Termamyl® SC* (FDA-GRASP 3G002612). The *Termamyl® LC* enzyme was constructed by splicing in the N-terminal region of the *B. amyloliquefaciens* α-amylase sequence and changing the DNA coding sequence for other specific amino acid residues to increase stability at low pH, low calcium concentration, and high temperature (FDA-GRN 22). The *Termamyl®* enzymes have been developed from gene bank nucleotide E01158 through an intermediate molecule named M38570. The enzyme preparations are used in the food industry as a processing aid in the liquefaction of starch to produce syrup or in the alcohol industry to thin starch in distilling mashes.

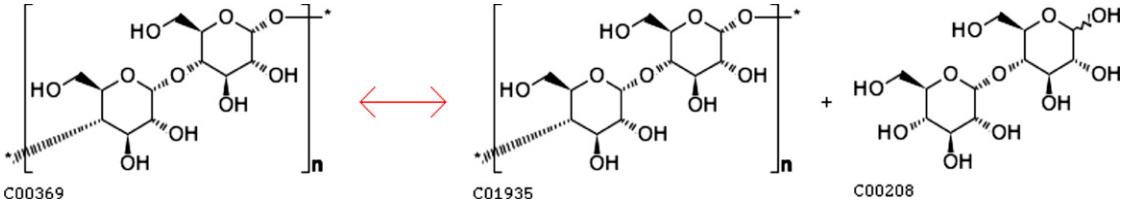
Based on the *Termamyl® LC* enzyme, another modified α-amylase, called Novozym® 28035, was developed for further increased stability at low pH, low calcium concentration, and high temperature. The Novozym® 28035 α-amylase enzyme has substitutions at four additional amino acid positions compared to its precursor, *Termamyl® LC* α-amylase, and its applications are equivalent (FDA-GRN 79).

Gene sequences from *Thermococcales archaebacteria* strains were used to create α-amylase BD5088 (FDA-GRN 126). The α-amylase designated BD5088 is a hybrid enzyme derived from three wild-type archaeal α-amylases. The hybridized enzyme was selected for commercialization owing to its stability and activity at low pH, high temperature, and low calcium concentration. Alignments of the thermophilic archaeal α-amylase amino acid sequences demonstrate at least 85% homology to BD5088 α-amylase. α-Amylase BD5088 was developed to be used in the hydrolysis of edible starch to produce starch hydrolysis products, including glucose syrup, high-fructose corn syrup (HFCS), and crystalline glucose (dextrose), and distilled ethanol for food and beverage use (FDA-GRN 126).

2. β-Amylases

β-Amylases (EC 3.2.1.2) or maltogenic-amylases act on the non-reducing end of starch, glycogen, and related polysaccharides and oligosaccharides to cleave β-maltose (two glucose units) by inversion. β-Amylase is intrinsic to bacteria and plants, where during the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. The term 'beta' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolyzed (KEGG R02112):

Starch <=> Maltodextrin + Maltose



3. Maltogenic Amylase

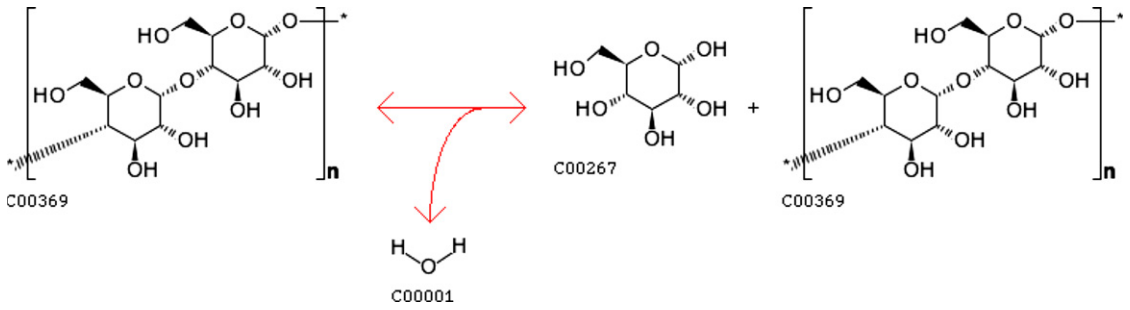
Maltogenic amylase (EC 3.2.1.133) is an exo-acting enzyme catalyzing the hydrolysis of α-1,4-glucosidic linkages in amylose, amylopectin, and related glucose polymers. α-Maltose residues are successively

removed from the non-reducing end of the starch or related polysaccharides and oligosaccharides polymers until the molecule is degraded or, in the case of amylopectin, a branch-point is reached (WHO Food Additives Series 40). Maltogenic amylase can be produced by submerged fermentation of a non-pathogenic and non-toxicogenic strain of *Bacillus subtilis*, which contains the *amyM* gene from *Bacillus stearothermophilus* coding for maltogenic amylase (WHO Food Additives Series 40; Diderichsen and Christiansen, 1988) (FDA-GRASP 7G0326).

4. γ-Amylases

γ-Amylases (EC 3.2.1.3) hydrolyze terminal 1,4-α-D-glucosidic bonds successively from non-reducing ends of the chains, releasing β-D-glucose. Most forms of the enzymes can rapidly hydrolyze 1,6-α-D-glucosidic bonds when the next bond in the sequence is 1,4, and some preparations of this enzyme hydrolyze 1,6- and 1,3-α-D-glucosidic bonds in other polysaccharides (KEGG R01790):

Starch + H_2O <=> alpha-D-Glucose + Starch



γ-Amylases or glucoamylases are only found in fungi (KEGG, OrthoDB), and unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3.

A glucoamylase from *Trichoderma reesei* is overexpressed in the same organism, *T. reesei*. The production strain has been genetically modified by inactivation of several cellulase genes to facilitate the overexpression of the native *T. reesei TrGA* gene for the production and secretion of recombinant glucoamylase enzyme.

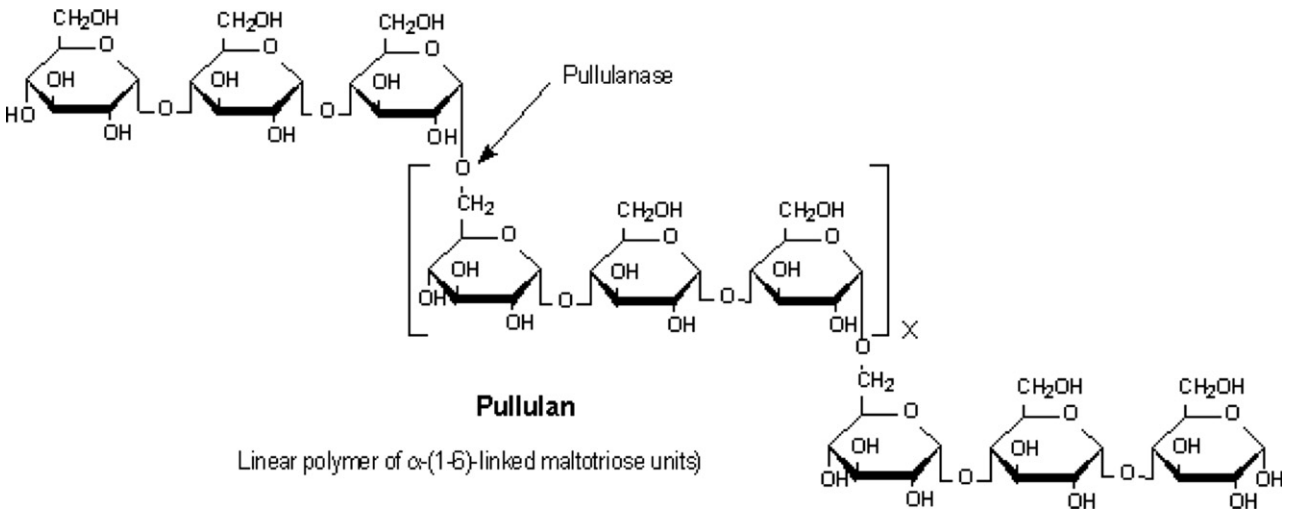
Two expression cassettes containing the *T. reesei cbh1* promoter and terminator flanking the *glal* DNA encoding the *T. reesei* glucoamylase are integrated into the genome of the final production strain, designated as 70H2-TrGA #32-9 (FDA-GRN 372).

The derived glucoamylase is used to saccharify liquefied corn starch for the manufacture of corn sweeteners such as HFCS. It maximizes the conversion of starchy substrate to fermentable carbohydrates. It enhances the extraction and saccharification of starch (mashing) from malted cereal, cereal, and other plant sources (including barley, corn, wheat, rye, milo, rice, tapioca, and potatoes). The resultant process liquors (worts) are fermented, typically by a yeast, to produce ethanol (and sometimes organic acids). *Trichoderma reesei* glucoamylase is also utilized in lactic acid manufacture processes similar to those used in potable alcohol manufacture (FDA-GRN 372).

5. Pullulanases

Pullulanases (EC 3.2.1.41) are specific kinds of glucanases, amylolytic exoenzymes, that degrade pullulan, amylopectin, glycogen, and α- and β-limit dextrins of amylopectin and glycogen. They are produced as extracellular, cell surface-anchored lipoproteins by Gram-negative bacteria. Type I pullulanases specifically attack α-1,6 linkages, while type II pullulanases are also able to hydrolyze α-1,4 linkages. Pullulanases cleave the branches of amylopectin molecules in starch to produce chains of amylase and are therefore called

debranching enzymes (Lee and Whelan, 1972). Maltose is the smallest sugar that they can release from an α-1,6 linkage:



(Singh et al. 2008)

Pullulanases are used predominantly in conjunction with other enzymes to enhance the saccharification of starch into glucose and glucose syrup. They are occasionally applied as a baking enzyme or in the alcoholic beverage industry, where they increase the amount of fermentable sugars available for conversion into alcohol (FDA-GRN 20). Pullulanases are produced through fermentation, utilizing cultures of bacteria (*Bacillus* and *Klebsiella*) or fungi (*Trichoderma*).

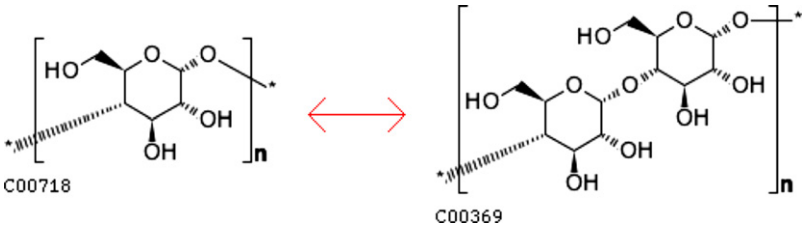
One specific recombinant *Bacillus naganoensis* pullulanase is expressed in *Bacillus subtilis* (FDA FDA-GRN 20). *Bacillus subtilis* is also utilized to express *Bacillus acidopullulyticus* pullulanase (FDA FDA-GRN 205). A third pullulanase derived from *Bacillus deramificans* is expressed in *B. licheniformis* (FDA FDA-GRN 72).

6. Maltotetraose-Forming Amylases

Maltotetraose-forming amylases (EC 3.2.1.60) are used to delay the staling process in bread and other baked goods and thereby extend the period for which the products have an acceptable eating quality. One maltotetraose-forming amylase, called G4-amylase, is applied as a processing aid in the starch industry. Heterologous G4-amylase is produced in *B. licheniformis* strain GICC03279, which is expressing the *mta* gene from *Pseudomonas saccharophila* (FDA-GRN 277). In order to improve thermostability during baking, the wild-type DNA sequence of the *P. saccharophila PS4* gene was altered by removing the C-terminal starch-binding domain and changing 16 out of the remaining 429 amino acids of the catalytic core (FDA-GRN 277). By introducing these mutations, the temperature stability and baking performance of the enzyme are increased, making this G4-amylase far better suited to anti-staling applications than the wild-type maltotetraohydrolase enzyme from *P. saccharophila.*

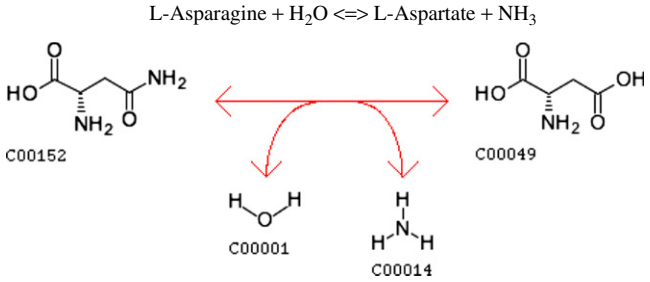
7. A Branching Glycosyltransferase

A branching glycosyltransferase (EC 2.4.1.18) from *Rhodothermus obamensis* produced in *B. subtilis* is used as an enzyme in the starch industry to obtain dextrins with improved physical properties, such as higher solubility, lower viscosity, and reduced retrogradation (FDA-GRN 274). The improved dextrin preparation can be used in soups, sauces, dried instant food, low-fat products, and soft drinks. The branching glycosyltransferase catalyzes the transfer of a segment of a 1,4-α-D-glucan chain to a primary hydroxyl-group in a similar glucan chain to create 1,6-α-linkages and increase the number of branched points. It converts amylose into amylopectin (KEGG R02110):



D. Asparaginase

Asparaginase (EC 3.5.1.1) converts the amino acid asparagine into aspartic acid (KEGG R00485):



Asparagine is a precursor of acrylamide, a suspected carcinogen. In the process of browning during the baking, frying, and grilling of starchy food, acrylamides are formed at temperatures higher than 100°C through the Maillard reaction. Through the addition of asparaginase, asparagine becomes unavailable and acrylamide contents may be reduced by up to 90% in processed food. The nutritional value, taste, and browning of the product remain unaffected (Pruser and Flyn, 2011).

Asparaginase preparations are applied to L-asparagine- and carbohydrate-containing foods that are heated above 120°C, such as bread (e.g. tin bread, buns and rolls, French sticks, variety breads like multigrain types of bread, raisin bread, biscuits, and crackers), other cereal-based products (e.g. cakes, Swiss rolls, Dutch honey cake, and breakfast cereals), potato-based products (e.g. French fries and potato chips), and reaction flavors (FDA-GRN 214).

Asparaginase preparations are available under the brand names *Acrylaway*® and *PreventASe*™. *PreventASe* is manufactured with the aid of the genetically modified mold *Aspergillus niger* transgenic for its own asparaginase *aspA* gene under the regulation of a *glaA* promoter for enhanced expression of the 378 amino acid enzyme. In the recipient *A. niger* strain GAM-53 seven glucoamylase gene loci were removed by replacement with plug sites. The major protease *pepA* gene, as well as the major amylases *amyA* and *amyB* were deleted, enhancing the strain's capacity to secrete proteins (FDA-GRN 214).

Acrylaway® is produced in the mold *Aspergillus oryzae*, carrying its own transgenic asparaginase gene under the control of the *Pna2/tpi* promoter for enhanced expression (FDA-GRN 201). The *A. oryzae* host strain BECh2 was modified to eliminate TAKA-amylase secretion and neutral metalloproteinase I by genetic deletion. In addition, cyclopiazonic acid and Kojic acid synthesis was eliminated by gamma- and ultraviolet-irradiation (FDA-GRN 201).

E. Aspartic Proteinase

Aspartic proteinase or mucorpepsin (EC 3.4.23.23) hydrolyzes proteins, favoring hydrophobic residues at P1 and P1′. It does clot milk but does not accept Lysin at P1, and hence does not activate trypsinogen.

Aspartic proteinase cloned from *Rhizomucor miehei* and produced in *A. oryzae* is used as a milk clotting enzyme and approved for use in cheese production (21CFR 173.1 50). An aspartic proteinase subcloned from *R. miehei* and produced in *A. oryzae* strain IFO 4177 is used as a tenderizing agent under the trademark NovoCarne™. The recipient *A. oryzae* strain IFO 4177 is the well-known genetically modified industrial production strain from the Institute for Fermentation, Osaka, Japan. In this expression plasmid the aspartic proteinase is under the control of the *A. oryzae* TAKA amylase gene promoter (FDA-GRN 34). The

enzyme is applied in liquid form by injection into the meat followed by tumbling to enhance distribution. It can also be mixed with the marinade for tumbling. NovoCarne Tender has a narrow specificity acting only on the myofibrillar proteins, not the connective tissue proteins. NovoCarne Tender also expresses self-limiting hydrolysis of myosin. These factors prevent NovoCarne Tender from overtenderizing the meat (FDA-GRN 34).

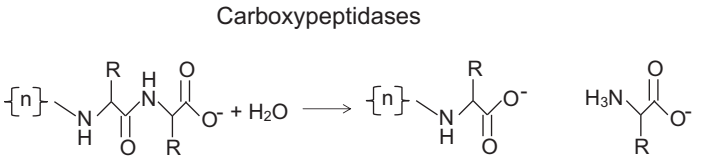
F. Aspergillopepsin I/Acid Fungal Protease

Acid fungal protease, synonymous with aspergillopepsin I (EC 3.4.23.18), is a protease hydrolyzing protein with broad specificity.

The gene for *Trichoderma reesei* acid fungal protease is overexpressed in the same microorganism, *T. reesei.* The DNA encoding the *T. reesei* mature secreted acid fungal protease was fused to the DNA encoding the *T. reesei* CBHI signal peptide, which enhances yield and secretion. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei* cellobiohydrolase I gene, to optimize overexpression. The isolated acid fungal protease is used in corn steeping, alcoholic beverage manufacture to increase free amino acids nitrogen or reduce foaming, dehazing apple juice, and degumming of membranes during orange juice manufacture (FDA-GRN 333).

G. Carboxypeptidase

Carboxypeptidases belong to the subclass of peptidases hydrolyzing polypeptide chains at the C-terminal end. They are also called exopeptidases (EC 3.4.11.x−3.4.19.x):



The technological function of carboxypeptidase is to release C-terminal amino acids from proteins and peptides present in various foods such as milk (casein, whey) and meat, in order to aid in and/or speed up the development of flavors during ripening.

A serine-type carboxypeptidase enzyme preparation is derived from genetically modified *Aspergillus niger* cultures and will be marketed under the trade name Accelerzyme® CPG. The *A. niger* host strain ISO-528 was derived from strain GAM-53 by deletion of seven loci of the glucoamylase gene *glaA*, which were replaced by so-called 'plug sites'. These *ΔglaA* plug sites allow for the site-directed insertion of expression cassettes. In addition, the *pepA* gene coding for the major protease was inactivated and the *amyA* and *amyB* genes encoding the major amylases were deleted. The strain also has an improved capacity to secrete proteins, which were selected by classical mutation breeding (FDA-GRN 345).

The carboxypeptidase expression cassette contains *A. niger*-derived DNA, starting with the *glaA* promoter, followed by the entire *pepG* genomic sequence encoding the carboxypeptidase protein, and 3′ flanked by the *gluA* terminator sequence. The *gluA* terminator sequence ensures efficient termination of *pepG* gene transcription and targeting of the expression unit to the *ΔglaA* loci (FDA-GRN 345).

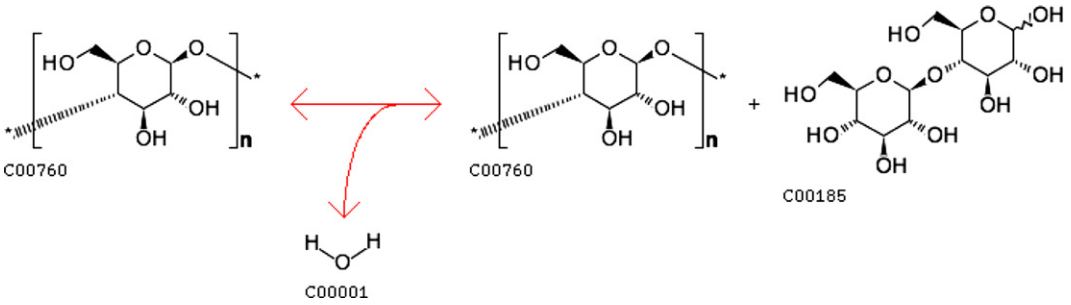
This Accelerzyme CPG carboxypeptidase preparation is to be used in cheese, enzyme-modified cheese, and fermented meat. In cheese production the enzyme is added to milk, together with the lactic acid bacteria, enabling the enzyme to act on the proteins present throughout the cheese and to release the amino acids as precursors for flavor components. In this way, the same types of flavor components are developed as during regular ripening, but the speed of the flavor development is accelerated, resulting in a shorter ripening period of the cheese, as well as a debittering during the ripening process. In fermented meat, carboxypeptidase can also be used to speed up the development of flavor during ripening. Fermented dry meat is produced by cutting and mixing fresh pieces of meat and bacon together with lactic acid bacteria. The mixture is filled in foil or natural pig-intestine, and left to dry and ferment in a temperature- and moisture-controlled ripening cell. During the ripening period of 3 weeks, the moisture decreases by evaporation, the pH decreases because

the lactic acid bacteria form lactic acid, and the taste develops because of flavor components formed by the lactic acid bacteria. By adding carboxypeptidase together with the lactic acid bacteria during cutting and mixing of the meat and bacon, amino acids are released that stimulate the lactic acid bacteria in their formation of flavor components. As a result, the meat reaches the desired taste after 22 instead of 28 days (FDA-GRN 345).

H. Cellulase

Cellulase (EC 3.2.1.4) is an enzyme responsible for the endohydrolysis of 1,4-P-D-glucosidic linkages in cellulose, lichenin, and cereal P-D-glucans. In addition, cellulases will hydrolyze the 1,4-linkages in P-D-glucans also containing 1,3-1inkages (KEGG R02886):

Cellulose + H_2O <=> Cellulose + Cellobiose



Cellulase enzyme preparations can be used to break down cellulose in a wide variety of cellulose-containing food products, such as the breakdown of cellulose in citrus products or other fruits used in juice and wine production, removal of fiber from edible oil press cakes, increase in starch recovery from potatoes and other starch sources, extraction of proteins from leaves and grasses, tenderizing fruits and vegetables prior to cooking, extraction of essential oils and flavoring material from plant materials, treatment of distiller's mash, extraction of green tea components, and other uses (FDA-GRN 292).

A cellulase enzyme preparation is derived from a genetically modified fungus. *Myceliophthora thermophila.* Additional copies of the *M. thermophila* cellulase gene *eg5* are incorporated into the chromosome of the *M. thermophila* recipient. These additional copies are under the control of the *cbh1* gene promoter, resulting in a hyperproducing cellulase strain (FDA-GRN 292).

I. Cyclodextrin Glucanotransferase

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) is a unique enzyme capable of converting starch or starch derivatives into cyclodextrins via the cyclization reaction. It cyclizes part of a 1,4-α-D-glucan chain by formation of a 1,4-α-D-glucosidic bond (KEGG EC 2.4.1.19).

The enzyme first cleaves the α-1,4-glycosidic bond between the residues bound at subsites +1 and −1, resulting in a covalent intermediate. The linear chain of the intermediate assumes a cyclic conformation, which is the circularization step. Subsequently, an α-1,4-glycosidic bond is reformed with the terminal 4-hydroxyl group of the intermediate. The catalytic residues involved in bond cleavage are Asp^{229} and Glu^{257} (numbering in *B. circulans* cyclodextrin glucanotransferase) (Figure 13.14) (Li *et al.*, 2007).

As a result, cyclodextrins (Schardinger dextrins) of various sizes are formed reversibly from starch and similar substrates. Linear maltodextrins can also be disproportionated without cyclizing. Cyclodextrins are cyclic α-1,4-glucans composed of between six to over 100 glucose-unit cyclodextrins, the most common forms being α-, β-, and γ-cyclodextrins. The enzymatic production of cyclodextrin from starch mediated by cyclodextrin glycosyltransferase results in a mixture of cyclodextrins α-, β-, and γ-, consisting of six, seven, and eight glucose units, respectively (Figure 13.15) (Li *et al.*, 2007).

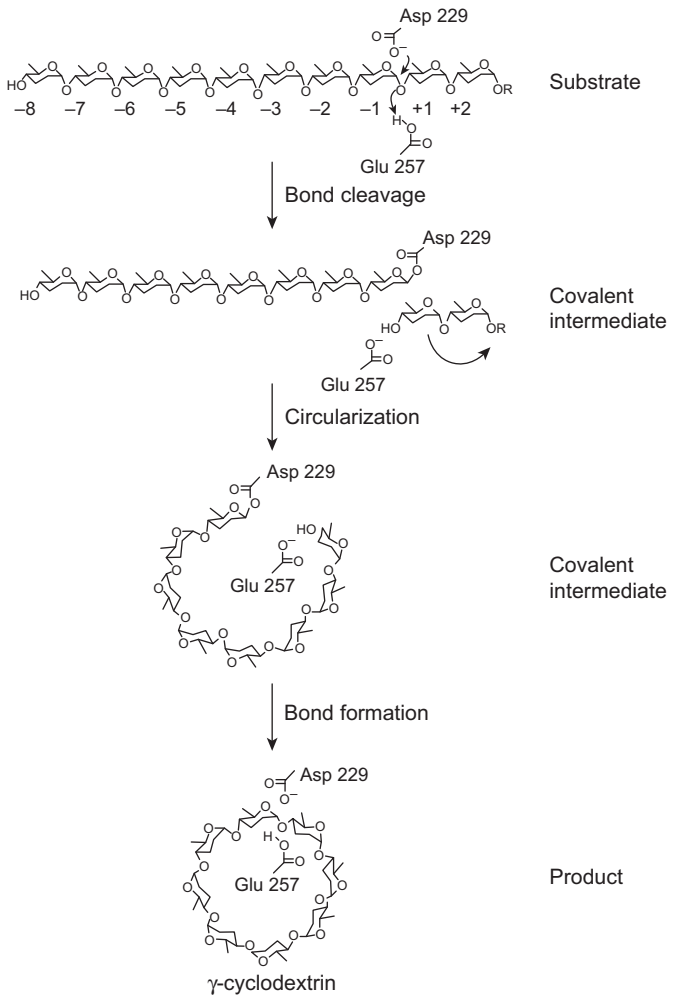


FIGURE 13.14 Circularization of starch derivatives into cyclodextrins catalyzed by cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19). *(From Li et al., 2007.)*

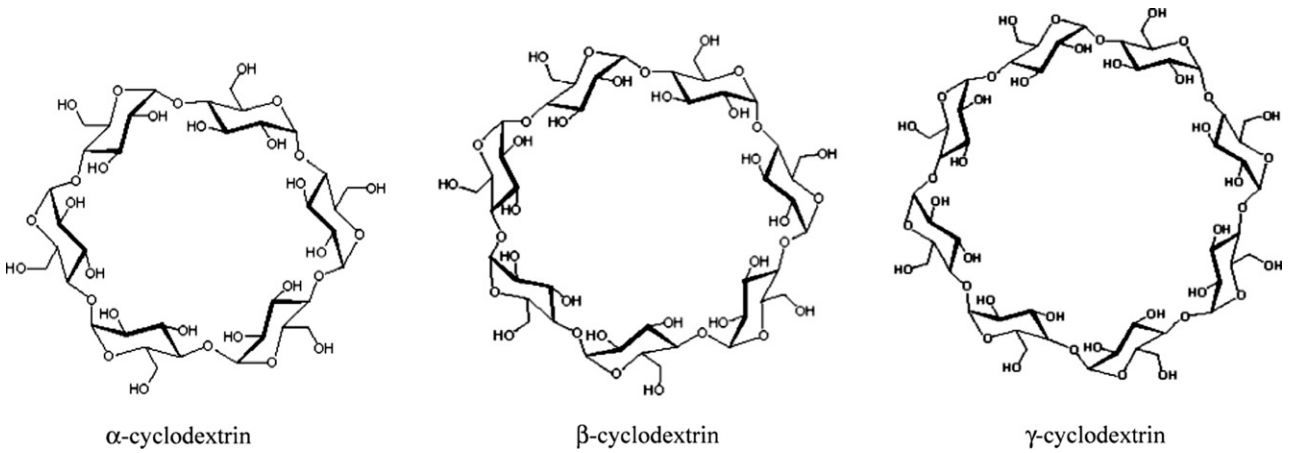


FIGURE 13.15 The most common forms of circular cyclodextrins. *(From Li et al., 2007.)*

The circular molecules of α-, β- and γ-cyclodextrin are shaped like a hollow truncated cone or torus. Because the hydrogen atoms and the oxygen atoms of the glycosidic bonds are facing the inner side of the torus, while the hydroxyl groups are located on the outer side, cyclodextrins have a hydrophobic cavity and, at the same time,

a hydrophilic outer surface which makes them water soluble. The hydrophobic cavity enables cyclodextrins to form inclusion complexes with a variety of organic compounds. The diameter of the cavity provides for a certain selectivity of the complexation of 'guest' molecules, i.e. the bigger ring of the eight-membered γ-cyclodextrin can accommodate a wider variety of guest molecules than the smaller rings of α- and β-cyclodextrins. Large guest molecules may complex with more than one cyclodextrin molecule (Tsoucaris *et al.*, 1987). The formation of an inclusion complex with a guest molecule is the basis for many applications of cyclodextrins in food, cosmetics, and pharmaceutical preparations (Li *et al.*, 2007).

Cyclodextrins can be used to increase the dietary fiber content of solid, semiliquid and liquid foods. Owing to their ability to form complexes with certain organic molecules, cyclodextrins can fulfill certain food-technological functions, e.g. as a flavor adjuvant; coloring adjunct; or a carrier, stabilizer, or solubilizer of certain vitamins (e.g. retinol acetate, vitamins K_1 and C, riboflavin, and β-carotene) and fatty acids (FDA-GRN 155). They are generally regarded as stabilizers, emulsifiers, carriers, and formulation aids in foods (FDA-GRN 64).

Cyclodextrins are produced using cyclodextrin glucanotransferase preparations. In the USA these preparations are produced in a recombinant strain of *E. coli* K12, which expresses the gene coding for the α-CGTase from *Klebsiella oxytoca* (FDA-GRN 155). The enzyme is completely removed from the final cyclodextrin products (FDA-GRN 155, FDA-GRN 64).

J. Chymosin

Chymosin (EC 3.4.23.4) is a peptidase with broad substrate specificity. It is specifically known for its ability to clot milk by cleaving at the Phe^{105}—Met^{106} bond in κ-casein (Gilliland *et al.*, 1991) and is therefore essential in the production of firm cheeses. Heterologously produced chymosin substitutes for the traditional preparations from calf stomach.

Bovine (*Bos taurus*) chymosin B is produced in submerged fermentation of *Trichoderma reesei*. Specifically, DNA encoding the prochymosin portion of the chymosin protein was synthesized using the preferred codon usage for *T. reesei* without changing the encoded amino acid sequence. The synthetic chymosin B gene is under the regulation of a native *T. reesei* promoter and terminator. A second expression cassette in the transforming DNA encodes for a *T. reesei* chaperonin that resides in the endoplasmic reticulum and is involved in folding of nascent secreted proteins fused to a different *T. reesei* promoter and terminator to control expression (FDA-GRN 230). *Trichoderma reesei* strain GICC03278 hosts the expression construct and the resulting preparation is known under the trade name Chymostar Supreme. The *T. reesei* strain GICC03278 is optimized by several gene deletions for the production of enzyme proteins.

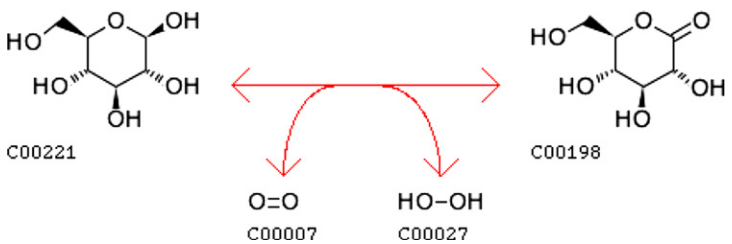
Bovine chymosin is produced as a fusion protein using the *A. niger* glucoamylase gene as a carrier molecule. A synthetic copy of the bovine chymosine having optimized codon usage and a change of serine-351 to threonine was introduced into *A. niger* var. *awamori*. Chymosin is released from the glucoamylase—prochymosin fusion protein by autocatalytic cleavage of the propeptide (Kappeler *et al.*, 2006).

Recombinant bovine chymosin transcribed from the synthetic bovine prochymosin gene is also produced in *E. coli*, *A. niger* var. *awamori* (Chy-Max®), and the yeast *Kluyveromyces lactis* (Maxiren®) (FDA 21 CFR 184.1685).

K. Glucose Oxidase

Glucose oxidase (EC 1.1.3.4) is an oxidoreductase catalyzing the oxidation of glucose to hydrogen peroxide (H_2O_2) and D-glucono-1,5-lactone, acting on CH—OH groups with oxygen as the acceptor (KEGG R01522):

beta-D-Glucose + Oxygen <=> D-Glucono-1,5-lactone + Hydrogen peroxide



(From Li et al., 2007.)

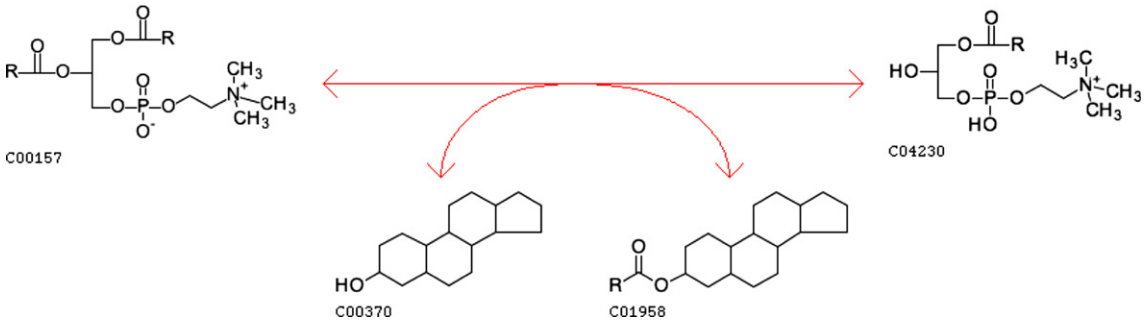
Glucose oxidase is used in the baking industry as a processing aid to strengthen gluten in dough systems. It contributes to the oxidation of free sulfhydryl units in gluten, whereby disulfide linkages are formed. This results in stronger, more elastic dough with greater resistance to mechanical shock, as well as better oven spring and larger loaf volume (FDA-GRN 106). Glucose oxidase is also utilized to remove D-glucose from egg white to prevent browning. It is used to eliminate oxygen from food packaging, such as the headspace above bottled and canned drinks, as well as reducing non-enzymatic browning in wines and mayonnaises.

Recombinant glucose oxidase is produced in the genetically modified *Aspergillus oryzae* strain BECh 2, which is amylase negative, alkaline protease (*alp*) negative, neutral metalloprotease I (*Npl*) negative, cyclopiazonic acid deficient, and kojic acid deficient. This microorganism carries the gene coding for glucose oxidase from *A. niger* under the control of the neutral amylase I (*Pna2ltpI*) promoter. Moreover, the 5′ non-translated part of this promoter has been replaced with the 5′ non-translated part of the *Aspergillus nidulans* triose phosphate isomerase (*TPI*) promoter (FDA-GRN 106). The resulting enzyme preparation is registered under the trade name Gluzyme®.

L. Glycerophospholipid Cholesterol Acyltransferase

Glycerophospholipid cholesterol acyltransferases (EC 2.3.1.43) catalyze the transfer of acyl residues, such as palmitoyl, oleoyl, and linoleoyl residues, to a variety of sterols (KEGG R02114):

Phosphatidylcholine + Sterol <=> 1-Acyl-*sn*-glycero-3-phosphocholine + Steryl-ester



A glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* is heterologously expressed in *Bacillus licheniformis*. The host organism is *B. licheniformis* Bra7, which was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene, and the native chloramphenicol resistance genes to make it suitable for expression of heterologous proteins. The gene coding for the *A. salmonicida* glycerophospholipid cholesterol acyltransferase was synthesized and codon optimized. One amino acid was changed and the synthetic gene is under the regulation of a native *B. licheniformis* promoter and terminator. Integration was achieved through homologous recombination into the *B. licheniformis* chloramphenicol resistance gene (*catH*), which functions for selection, chromosomal integration, and cassette amplification. The *catH* genetic sequences surround the expression cassette with upstream and downstream sequences. Part of the upstream *catH* sequence (called 5′ repeat) is present twice on the plasmid to allow amplification of the expression cassette on the chromosome (FDA-GRN 265).

The effectiveness of the *B. licheniformis* glycerophospholipid cholesterol acyltransferase is based on its effects on the cell membrane by transferring acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols, and other smaller primary alcohols. The acyl groups transferred are mainly C14 to C18 fatty acids: myristic, palmitic, stearic, oleic, linoleic, and linolenic acids. Cholesterol and other sterols accept the transferred acyl groups to become cholesterol-esters and sterol-esters. Fatty alcohols (defined as C12 and larger alcohols) can also be esterified. The reaction products formed depend on the substrate(s), but generally consist of lysophospholipids, cholesterol ester of C14 to C20 fatty acids and sterol esters of C14 to C20 fatty acids (campesterol, stigmasterol, betasitosterol, 5-avenasterol, and 7-stigmasterol).

The enzyme preparation is used in egg yolk and whole eggs, in processed meats, in degumming of vegetable oils, in milk products such as cheese, and in bakery products containing eggs, such as cake products. The trade name is FoodPro™ LysoMaxa Oil (FDA-GRN 265).

Egg yolk is well used in the food industry owing to its emulsification properties. Approximately 30% of the lipid in egg yolk is phospholipid, which contributes to egg yolk's emulsification properties. In many foods including mayonnaise, sauces, dressings, and cakes, the emulsification properties of egg yolk are exploited. For some food applications, however, the emulsification properties of egg yolk are not sufficient to obtain a homogeneous product without separation. In mayonnaise, for instance, pasteurization of the product at high temperatures causes the product to separate. The enzyme preparation will be used to modify phospholipid to lysophospholipid and cholesterol-ester in egg yolk. Product separation at high-temperature pasteurization can be avoided using enzyme-modified egg yolk for production of mayonnaise. In cakes, enzymatically modified egg yolk gives a softer and more tender crumb.

The enzyme preparation is used in processed meat products. It improves the emulsification of processed meat products and contributes to better consistency and reduced cooking loss. The enzyme preparation added to processed meat converts meat phospholipids to lysophospholipids.

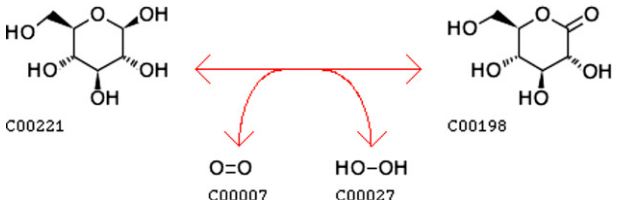
Crude vegetable oils such as soybean oil contain around 12% phospholipids, which are removed from the oil during the refining process, in order to improve the quality and prevent sedimentation in the oil. The removal of phospholipids is conducted by a degumming process during the oil-refining process. The degumming can be conducted by chemical or enzymatic means. In the degumming process the enzyme converts phospholipids to lysophospholipids which are more water soluble and can be removed from the oil by washing with water. Enzymatic hydrolysis of phospholipids is a gentler process than chemical degumming, which needs more acids and alkalis. Furthermore, degumming with the enzyme will produce fewer effluents. During the degumming process the enzyme catalyzes the transfer of fatty acids form phospholipids to phytosterols in the oil during the formation of phytosterol esters. Phytosterols are normally removed by deodorization during oil refining, but when the enzyme preparation is used, the phytosterols are converted to phytosterol esters; these esters are not removed during the refining process because of the lower volatility of the sterol esters. Phytosterol esters are not unknown constituents in vegetable oil because a smaller part of phytosterol exists naturally in the form of esters.

The enzyme preparation in milk products contributes to increased yield during cheese production. The enzyme added to milk converts milk phospholipids to lysophospholipids, enhancing emulsification properties and increasing cheese yield by entrapping more lipid in the cheese curd (FDA-GRN 265).

M. Hexose Oxidase

Hexose oxidase (EC 1.1.3.5) catalyzes the oxidation of various monosaccharides and oligosaccharides (principally glucose, but also maltose, lactose, D-galactose, D-mannose, and cellobiose) with oxygen to produce lactones and hydrogen peroxide (KEGG R01522):

beta-D-Glucose + Oxygen <=> D-Glucono-1,5-lactone + Hydrogen peroxide



The main application of hexose oxidase is in bread making, to increase dough strength and bread volume. The enzyme acts in a similar way to glucose oxidase for this purpose, where the produced hydrogen peroxide acts as an oxidant with other food components. However, it has added advantages, since it acts on a wider range of substrates. Other applications in the food industries are in cheese and tofu manufacture, where it aids curd formation, limiting undesirable browning by limiting Maillard reactions in food, and as an oxygen scavenger during the production of dressings and sauces. It can also be used in the pasta and noodle manufacturing industries, where its use strengthens the structure of the dough, resulting in reduced loss of starch and protein on cooking and a firmer bite and better texture (FSANZ A475).

It has been known for some time that hexose oxidase can be extracted from a number of red algae. However, because of the difficulty in recovering the small amounts of the enzyme from such algae, little use has been made of

its properties. This has been overcome in recent times by using recombinant DNA technologies to produce greater quantities of enzyme to enable more complete characterization. Such techniques have allowed industrial processes to be used to produce commercially viable enzyme preparations.

The gene encoding for hexose oxidase has been isolated from the alga *Chondrus crispus* and inserted into the yeast host *Hansenula polymorpha*. The plasmid used in this construction was created from the *E. coli* plasmid pBR322 by insertion of the *URA3* gene (encoding oritidine-5′-phosphate decarboxylase) from *S. cerevisiae*, a native *H. polymorphu* promoter and terminator, and the gene encoding for hexose oxidase from *C. crispus*. Furthermore, the genes coding for ampicillin resistance (*Apr*) and tetracycline resistance (*TCr*) in the original pBR322 have been removed. The subsequent organism produces the enzyme in commercial quantities during a submerged fermentation process (FSANZ A475, FDA-GRN 238).

N. Ice Structuring Protein

Ice structuring proteins are widely distributed in nature, for example in coldwater fish, vegetables, grains, lichens, and bacteria. Ice structuring proteins bind to ice and help organisms to cope with very cold environments, both by lowering the temperature at which ice crystals form and by modifying the size and shape of the ice crystals so that the ice is less damaging to tissues (Hall-Manning *et al.*, 2004; EFSA 768, 2008).

Ice structuring protein type III was originally isolated from the blood of *Macrozoarces americanus*, the ocean pout. The ice structuring protein from this fish consists of 12 isoforms that can be separated by high-performance liquid chromatography (HPLC). Isoform HPLC-12 gives the largest peak and is the most functionally active in *in vitro* studies on ice structuring (EFSA768, 2008). This protein is specifically identified by accession number P19614 in the Swiss Prot database. It has a molecular weight of 7.027 kDa, is not glycosylated, is heat stable, is stable over a pH range of 2−12, and consists of the following 66 amino acids:

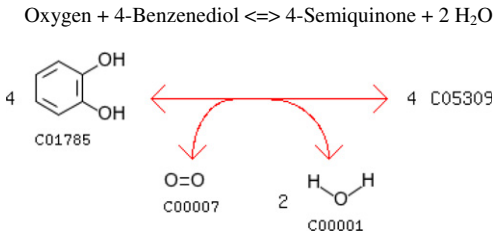
NQASVVANQL IPINTALTLV MMRSEVVTPV GIPAEDIPRL VSMQVNRAVP LGTTLMPDMV KGYPPA

This *M. americanus* ice structuring protein isoform is prepared by fermentation of a genetically modified strain of food-grade baker's yeast *S. cerevisiae* in which a synthetic gene for the ice structuring protein has been inserted into the yeast's genome. The ice structuring protein expressed by the yeast has the same amino acid sequence as that from the ocean pout but the nucleotide sequence was constructed to favor codon usage in yeast to maximize expression. The protein is expressed and secreted into the growth medium (EFSA768, 2008, FDA-GRN 117). The ice structuring protein produced in the recombinant yeast strain is a mixture of 60% unglycosylated and 40% glycosylated protein. However, only the unglycosylated ice structuring protein is able to bind to ice crystals and alter the ice structure.

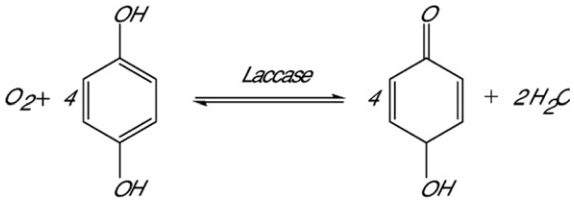
Ice structuring protein is used in the production of edible ice products, e.g. ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts, and any similar products such as iced smoothies. It allows for the production of products lower in fat, sugar, and calories. From 2003 to 2007 more than 470 million ice structuring protein-containing edible ice products were sold in the USA and 47 thousand liters of ice structuring protein containing ice cream was sold in Australia and New Zealand (EFSA768, 2008).

O. Laccase

Laccases (EC 1.10.3.2) are a group of multicopper oxidoreductases acting on both *o*- and *p*-quinols, and often acting also on aminophenols and phenylenediamine (KEGG R00083):

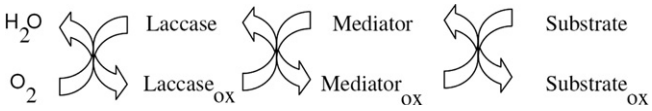


Alternatively:



The semiquinone may react further either enzymatically or non-enzymatically. Laccases have a wide range of phenols and other substrates with the concomitant reduction of oxygen to water. Reactions between laccases and phenols are commonly found in nature. One prominent example is the oxidation of phenols in fruits and vegetables by laccases to form brown polymers, which is known as the bruising or enzymatic browning of fruits and vegetables.

In breath-freshening products such as breath mints, chewing gum, toothpaste, and mouthwash, laccase facilitates a reaction of naturally occurring polyphenolic compounds in food. The resulting semiquinones react with odor-causing volatile sulfur compounds in the oral cavity to deodorize them, therefore removing bad breath (FDA-GRN 122):



(Kunamneni et al., *2008)*

Since laccases have the capability of catalyzing the oxidation and the polymerization of phenols and other substances, they are used in various applications such as in wine clarification, improved storage of beer, stabilization of fruit juices, dough improvement in bread making, and ethanol production. Fungal laccases are used to speed up the polymerization of polyphenols in the production of beverages such as beer, wine, and apple juice. In wine clarification, laccase is used to remove phenols from white grape must. Another potential application is oxygen removal during processing of foodstuffs, preventing oxidation of sensitive substances while preserving aromatic compounds or preventing the formation of off-flavors (FDA-GRN 122).

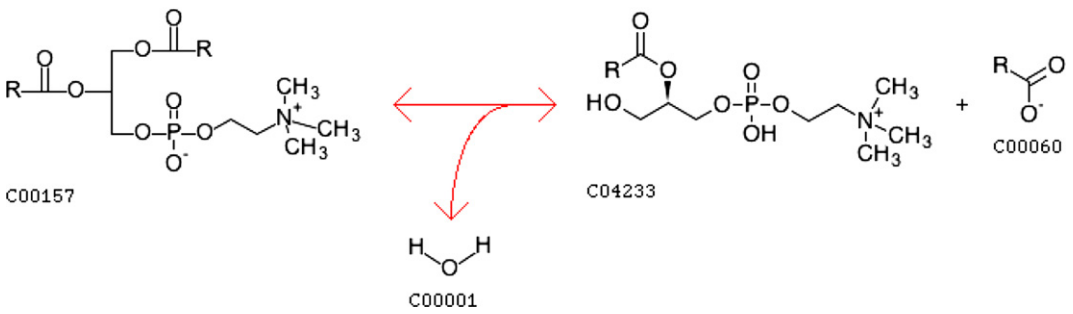
A transgenic laccase is produced by submerged fermentation of *Aspergillus oryzae* strain *How B711*, where the three resident TAKA amylase genes were removed by site-directed gene disruption and the expression plasmid for the encoding of a laccase from *Myceliophthora themophila* was inserted. Here, the laccase gene is under transcriptional control of the *A. oryzae TAKA*-amylase promoter and the *A. niger* glucoamylase terminator.

P. Lipases

1. Phospholipase A_1

Phospholipase A_1 (EC 3.1.1.32) hydrolyzes the *sn*-ester bond of diacylphospholipids to form 2-acyl-lysophospholipid and free fatty acid (KEGG R01314):

Phosphatidylcholine + H_2O <=> 2-Acyl-*sn*-glycero-3-phosphocholine + Carboxylate



Phosphatidylcholine, often referred to by the name lecithin, is composed of a glycerol backbone esterified to phosphocholine and two fatty acids, and is one of the major components of the phospholipid portion of the cell membrane.

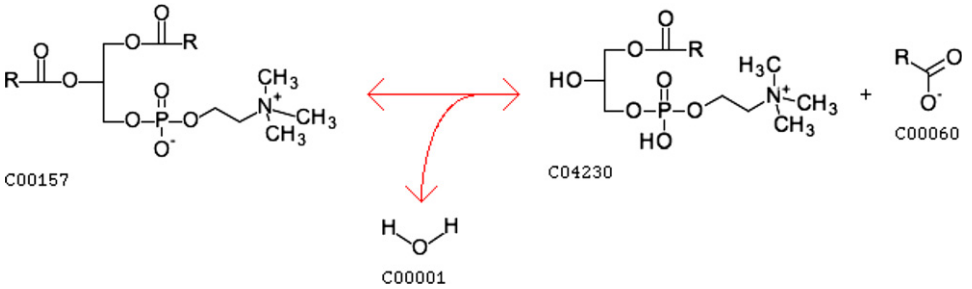
The *FvPLAl* gene from the fungus *Fusarium venenatum* is used for heterologous production of phospholipase A_1 in *A. oryzae* strain BECh 2. The expression cassette contains a double promoter element constructed from the *Pna2* TATA less neutral amylase II promoter from *A. niger*, fused to the Pna2/TPI unit, which is the neutral amylase II promoter from *A. niger* where the 5′ non-translated part has been replaced with the 5′ non-translated part of the *A. nidulans* triose phosphate isomerase (TPI) promoter. The *F. venenatum* phospholipase gene *FvPLAl* is flanked in the 3′ by the amyloglycosidase terminator of *A. niger* (FDA-GRN 142).

The enzyme preparation has the trade name Novozyme® 46016 and specifically acts on the fatty acid in position 1 of phospholipids. It is used in the dairy industry as a processing aid during cheese production, where it is added to the cheesemilk. The modified phospholipids in the cheesemilk have improved emulsifying properties and as such result in better production efficiency by keeping more of the original milk components in the cheese and reducing the loss of fat and other solids into the whey stream (FDA-GRN 142).

2. Phospholipase A_2

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of 3-*sn*-phospholipids exclusively at the 2-position, giving rise to the formation of 1-acyl-3-*sn*-lysophospholipids and free fatty acids (KEGG R01313):

Phosphatidylcholine + H_2O <=> 1-Acyl-*sn*-glycero-3-phosphocholine + Carboxylate



Although phospholipase A_2 is able to hydrolyze these phospholipids in their monomeric form, the enzyme is much more active when these substrates are organized in the form of micelles or lipid membranes. The presence of calcium ions is essential for the enzymatic activity of phospholipase A_2.

The genetically modified glucoamylase and protease negative *A. niger* strain ISO-502 is used as the host strain to express porcine phospholipase A_2. The coding sequence for the porcine phospholipase A_2 was derived from a pancreatic tissue cDNA library and integrated into a fusion protein. The coding sequences of the 498 N-terminal amino acids of the *A. niger* glucoamylase (*glaA*) gene are linked to the porcine phospholipase A_2 gene via a synthetic KEX2 proteolytic site. The expression of this construct is regulated by the *glaA* promoter and effective termination of the RNA is ensured by the 3′-flanking *glaA* terminator, both derived from the parental *A. niger* strain. The production strain, designated PLA-54, harbors multiple copies of the expression cassette, resulting in commercially attractive expression levels of the phospholipase A_2 enzyme (FDA-GRN 183).

The expression unit is translated into a glucoamylase—pro-phospholipase A_2 fusion protein. During secretion of this protein by the microbial cell, the endogenous kexine protease separates the pro-phospholipase A_2 from the glucoamylase part at the KEX2 proteolytic site. Also, the pro-part of the phospholipase A_2 is split off, resulting in the mature, active enzyme. The truncated secreted glucoamylase still has full enzymatic activity (FDA-GRN 183).

The heterologous porcine phospholipase A_2 hydrolyzes natural phospholipids present in foodstuffs, resulting in the formation of lysophospholipids. Lysophospholipids are surface-active agents with emulsifying properties to mimic the effects of chemical emulsifiers in food. During mixing and subsequent fermentation of bread dough, phospholipase A_2 hydrolyzes the phospholipids present in wheat flour mixes for bread making. The resulting lysophospholipids strengthen the dough to improve the dough mixing tolerance and machinability, as well as gas retention, to increase the loaf volume, crust crispiness, and crumb texture and softness. In egg-yolk-based fine bakery wares phospholipase A_2 hydrolyzes the phospholipids naturally present in the eggs that are part of the batter recipe. This contributes to the structure of batter-derived products such as sponge and pound cakes through efficient air incorporation and retention.

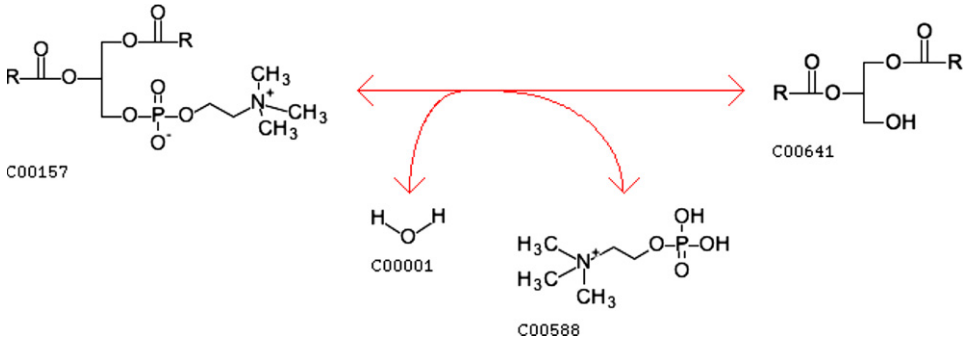
The addition of phospholipase A_2 to egg-yolk-based sauces and dressings significantly enhances the emulsifying properties, heat stability, and viscosity (FDA-GRN 183). The trade names for the spray-dried products are Bakezyme and Cakezyme (depending on the application) and the liquid product is sold under the trade name Maxapal A_2.

Another heterologous phospholipase A_2 is produced in the *Streptomyces violaceruber* strain AS-IO, carrying an expression cassette containing the gene encoding a phospholipase A_2 enzyme from *S. violaceruber* regulated by the *Streptomyces cinnamoneum* phospholipase D promoter and terminator. The enzyme preparation, called PLA2 Nagase, is used as a processing aid in egg yolk treatment or in hydrolyzing lecithin. The treated egg yolk or hydrolyzed lecithin has improved emulsifying properties and results in improved taste when added in food such as mayonnaise and/or a longer shelf-life of the product (FDA-GRN 212).

3. Phospholipase C

Phospholipases C (EC 3.1.4.3) catalyze the ester hydrolysis of a phospholipid to a diglyceride and choline phosphate (KEGG R01312):

Phosphatidylcholine + H_2O <=> 1,2-Diacyl-*sn*-glycerol + Choline phosphate



Bacterial Phospholipases C also act on sphingomyelin and phosphatidylinositol; that from seminal plasma does not act on phosphatidylinositol.

A phospholipases C encoding genetic fragment, designated as a BD1649 PLC gene, was isolated from a soil environmental DNA library and transferred into *Pichia pastoris* to create the strain DVSA-PLC-004. The specific DNA sequences used in the strain's construction include the phospholipases C encoding gene from an environmental library; linked to the *S. cerevisiae* α-mating factor secretion signal sequence; a fragment of the *P. pastoris* alcohol oxidase gene (3′ *P.p. AOXI);* a *Pichia pastoris* selectable marker gene, *HIS4* (histidinol dehydrogenase); and the well-characterized, non-coding *P. pastoris* regulatory sequences comprising the alcohol oxidase promoter (*AOXI*) and the transcriptional terminator from *AUXl* (FDA-GRN 204).

The enzyme preparation is used for the degumming of oils, the first step in this refining process designed to remove contaminating phospholipids or phosphatides that otherwise interfere with the processing of high-phosphorus oils such as soybean, canola, corn, and sunflower oils. BD 16449 phospholipase C catalyzes the hydrolysis of the phosphodiester bond linking the glycerol and phosphate moieties at the *sn*-3 position of glycerophospholids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid; for example:

$$\text{Phosphatidylcholine} + H_2O <=> 1,2\text{-Diacyl-}sn\text{-glycerol} + \text{Choline phosphate}$$

$$\text{Phosphatidylethanolamine} + H_2O <=> 1,2\text{-Diacyl-}sn\text{-glycerol} + \text{Ethanolamine phosphate}$$

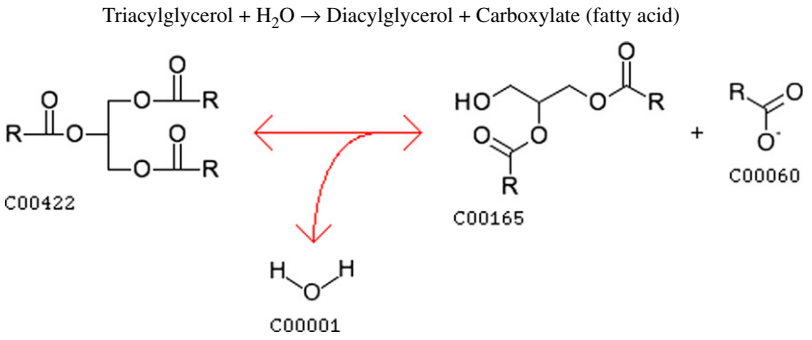
The products of the hydrolysis are diacylglycerol and water-soluble phosphate esters, resulting in the following benefits:

- reduction in the amount of gum phospholipids and overall gum mass
- reduction in the total phosphorus contained in the oil
- reduction in the total mass of neutral oil entrained in the phospholipid gum
- increase in the quantity of diacylglycerol contained in the oil.

Therefore, the oil yields are higher, and less bleaching is needed, resulting in lower usage of water and bleaching earth, and overall reduced use of environmental resources.

4. Triacylglycerol Lipases

Triacylglycerol lipases (EC 3.1.1.3) catalyze the hydrolysis of triglycerides, as well as phospholipids and galactolipids by acting on ester bonds (KEGG R01369):



Triglyceride lipases hydrolyze esters in an aqueous solution; however, they are also known to catalyze esterification of fatty acids and alcohols or rearrange fatty acids in glycerides under certain conditions where water content is low (FDA-GRN 43). Hence, the specificity of a lipase is not only determined by the species, but also depends on the reactants and the reaction conditions. For example, in some reactions a lipase shows 1,3 specificity, whereas in other reactions it functions as a non-positional lipase.

Rearrangement of fatty acids in glycerides can improve the physical and nutritional characteristics of the glyceride products. Lipases are applied in the fruit juice industry, baked goods, vegetable fermentation, and dairy products. Lipases have traditionally been used in the oils and fats industry, where they are utilized primarily to catalyze the cleavage of fatty acids from triglycerides. Lipases are used for degumming of edible oils by removing phospholipids. They can also be used to improve the emulsifying properties of ingredients (such as lecithin and egg yolk) during food processing. In bread making the enzymes improve dough stability and dough handling properties and subsequently bread volume and crumb homogeneity (FSANZ A569). There is a variety of heterologously produced triacylglycerol lipases in the food chain today and in the following section the most prominent will be discussed.

One recombinant triacylglycerol lipase enzyme preparation is produced by submerged fermentation using a selected strain of the yeast *Hansenula polymorpha* that carries the gene coding for a triacylglycerols lipase from the mold *Fusarium heterosporum*. The native gene was resynthesized with codon usage optimized for expression in yeast. In the expression cassette the synthetic gene encodes the same amino acid sequence as the native gene from *F. heterosporum* and it is under the control of a native *H. polymorphu* promoter and terminator (FDA-GRN 238, FSANZ A569). This specific enzyme preparation has the marketing name of GRINDAMYL™ POWERBake.

Transgenic triacylglycerol lipases produced in *Aspergillus niger* are marketed under the trade name Panamore™ for their ability to improve baking properties. In this specific case the *A. niger* host carries a gene coding for a pre-prolipase *lfs* gene which was constructed synthetically based on sequences of various *Fusarium* species. Expression is controlled by the glucoamylase *glaA* promoter and the 3′ flanking *glaA* terminator sequence from the parental *A. niger* (FDA-GRN 296).

A *Candida antarctica*-derived lipase is produced in the *A. niger* strain *MBinl18* which has been optimized for expression in heterologous proteins by deletion in the *pyrG* gene, interruption of the glucoamylase, acid-stable amylase, protease regulator, neutral amylase II, and neutral amylase I gene. The *C. antarctica* lipase is under the control of *Pna2/tpi*, the neutral amylase II promoter from *A. niger*, in which the 5′ non-translated part has been replaced with the 5′ non-translated part of the *Aspergillus nidulans* triose phosphate isomerase promoter. The amyloglycosidase terminator of *A. niger* completes this expression cassette. The enzyme preparation is marketed as Lipozyme® and is designated for the oils and fats industry to improve the physical and/or the nutritional properties of triglyceride products (FDA-GRN 158).

Cultures of *Aspergillus oryzae* are utilized to produce a variety of transgenic triacylglycerol lipases originating from the fungi *Fusarium oxysporum*, *Rhizomucor miehei*, and *Thermomyces lanuginosus* (previously known as *Humicola lanuginose*) (FSANZ A569, FDA FDA-GRN 43, FSANZ A402).

Cultures of the genetically modified *A. oryzae* strain H-1-52/c (synonym AI-11) are expressing the *T. lanuginosus* triacylglycerol lipase gene under the control of the *A. oryzae* TAKA amylase promoter and the *A. niger* glucoamylase

terminator sequence. Two enzyme preparations derived from these cultures are marketed; Lipozyme TL IM, for interesterification of bulk fats, or production of frying fats, shortenings, and margarine components; and Novozym 677 BG, for increased dough stability and larger volume of baked goods, improved crumb softness, as well as structure and whiter crumb in bread making (FDA-GRN 43).

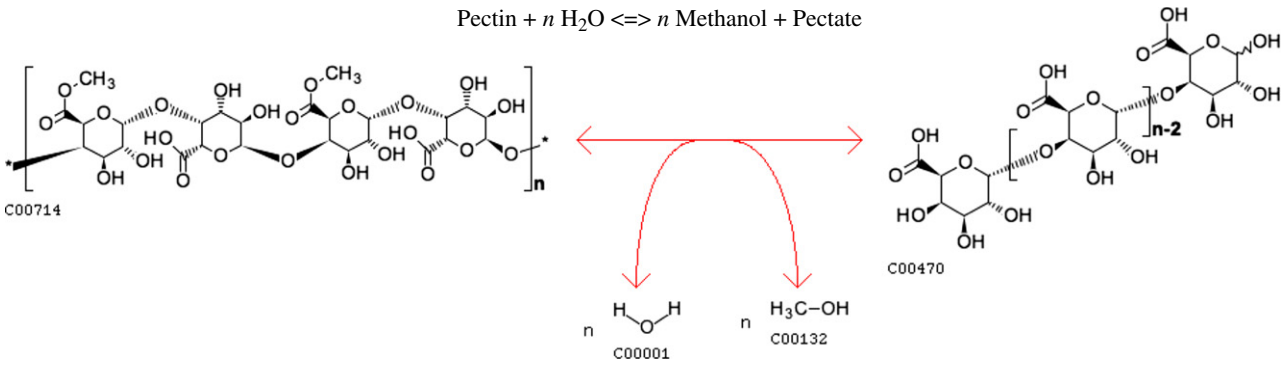
A triacylglycerol lipase from *F. oxysporum* is used for heterologous expression in *A. oryzae* strain *MStrl15*, which was genetically modified to be negative for amylase, alkaline protease, and neutral metalloprotease I. This expression cassette is under the control of the *A. niger* neutral amylase II gene promoter, which is fused to the 5′ non-translated leader of the *A. nidulans* triose phosphate isomerase gene. Termination of transcription is ensured by the terminator of the *A. niger* amyloglycosidase gene. Resulting enzyme preparations are marketed as Lipopan F for baking applications and Lecitase® Novo in the fats and oils industry for degumming of vegetable oil as well as for the hydrolysis of lecithin for altered emulsifying properties and modification of egg yolk (FDA-GRN 75).

The *R. miehei* triacylglycerol lipase expressed in *A. niger* is marketed under the trade name Palatase®, but unfortunately no information on the nature of the genetic constructs seems to be available in the public records (FSANZ A402).

A hybrid genetic construct was derived from the two fungi *T. lanuginosud* and *F. oxysporum* to yield a triacylglycerol lipase specifically acting on the fatty acid in position 1 in both triglyceride substrates and phospholipids (FDA-GRN 103). The hybrid protein is constituted of the 284 N-terminal amino acids from the *T. lanuginosud* lipase *TI1* gene fused to the 54 C-terminal amino acids of the *F. oxsporurn* lipase *FoL* gene. In addition, the sequence of the *T. lanuginosud* lipase was changed at three specific amino acid residues: glycine^{113} → alanine, aspartic acid^{118} → tryptophan, and glutamic acid^{121} → lysine. The preparations' trade names are Lipopan® H and Lecitase® Ultra, used for baking applications and in the fats and oils industry, respectively. In the baking applications, the activity towards both triglyceride and phospholipid substrates is utilized, while in the fat and oil applications, the activity towards the phospholipids (phosphatides, lecithins) will predominate as a result of specific processing conditions. The specificity of the lipase towards long-chain fatty acids in position 1 is utilized in baking applications, where the lower tendency to release short-chain fatty acids from primary ester bonds in triglycerides and phospholipids decreases the risk of off-flavor generation in baking formulae that contain butterfat. In fats and oils applications this enzyme has increased activity towards phospholipids and high efficacy when used for the modification of egg yolk or whole egg, hydrolysis of lecithin for altered emulsifying properties, and vegetable oil degumming (FDA-GRN 103).

Q. Pectinesterase

Pectinesterases (EC 3.1.1.11) catalyze the de-esterification of pectin into pectate and methanol. They hydrolyze the ester linkage between methanol and galacturonic acid in esterified pectin (KEGG R02362):



Pectin is one of the main components of the plant cell wall. In plants, pectinesterases play important roles in cell wall metabolism during fruit ripening. The enzymatic de-esterification of pectins results in a low methoxylated pectin, which in the presence of calcium ions forms a strong gel. The enzymatic conversion of high methoxylated to low methoxylated pectin makes a gel formation possible and may, in products such as jam and ketchup, render further addition of thickening agents unnecessary. Pectin esterase also firms fruit and vegetables after it is infused into the tissue (FDA-GRN 8).

Heterologous pectin esterase is produced by *Aspergillus oryzae* strain IF0 4177 carrying the gene coding for pectin esterase from *Aspergillus aculeatus*. The expression cassette consists of the *A. oryzae* TAKA amylase gene

promoter, followed by the *A. aculeatus* pectin esterase gene and the *A. niger* glucoamylase gene terminator sequence. The resulting enzyme preparation is marketed under the trade name Rheozyme™, used for the gelation of plant materials, thickening of plant preparations, hardening of fruits and vegetables, and controlled demethylation of high methoxylated pectins. It can be found in fruit preparations, compote, cider, jam, and tomatoes (FDA-GRN 8).

R. Pectin Lyase

Pectin is a heteropolysaccharide found in fruits with galacturonic acid and methanol as the main components, and some neutral sugars attached, such as D-galactose, L-arabinose, and D-xylose. Pectin molecules are formed by α-1,4-glycosidic linkages between the pyranose ring of D-galacturonic acid units. These galacturonan chains are interrupted at intervals by the insertion of 1,2-linked α-L-rhamnopyranosyl residues. The carboxyl groups of pectin are partially esterified with methanol, and the hydroxyl groups are sometimes partially acetylated.

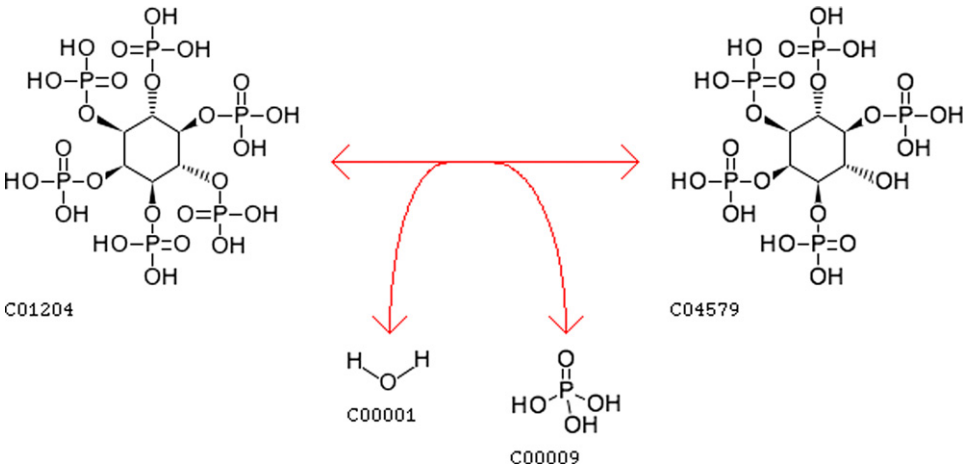
Pectin lyases (EC 4.2.2.10) cleave 1,4-α-D-galacturonan methyl ester to oligosaccharides with 4-deoxy-6-*O*-methyl-α-D-galact-4-enuronosyl groups at their non-reducing ends. Hence, the enzyme cleaves the methylated polygalacturonic acid backbone of esterified pectin by a transelimination reaction and the cleaved oligomers contain a 4,5-dehydrogalacturonic acid group at their non-reducing end.

A pectin lyase from *Trichoderma reesei* is produced in *Aspergillus niger*. The *T. reesei* pectin lyase gene (*pe/D*) in the recombinant *T. reesei* strain is under the control of the *cbhl* promoter. This pectin lyase is a highly specific endo-enzyme with the ability to cleave highly esterified polygalacturonic acid. However, only those glycosidic bonds adjacent to a methyl ester group are split. Pectin lyase therefore shows high activity only towards highly esterified pectins. The degree of esterification in water-soluble pectin is 65−98%. The cleavage of only a small number of glycosidic linkages causes a sharp decrease in viscosity. The decrease in viscosity has a favorable influence both on the rate of filtration and on the heat transmission coefficient during concentrate manufacture. This specific heterologous pectin lyase enzyme preparation is used for applications in the processing of fruits and vegetables where a fast reduction of viscosity is needed. In the processing of juices the yield and the coloring matter will increase (FDA-GRN 32).

S. Phytases

A phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is any type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate), an undigestible organic form of phosphorus that is found in grains and oil seeds, and releases a usable form of inorganic phosphorus (Mullaney *et al.*, 2000a, b). 4-Phytase, also named 6-phytase (EC 3.1.3.26), mediates the following reaction (KEGG R03372):

myo-Inositol hexakisphosphate + H_2O <=> Inositol 1,2,3,5,6-pentakisphosphate + Orthophosphate



A heterologous 6-phytase from *Peniophora lycii* is heterologously produced in *Aspergillus oryza* strain Pz-3 (FSANZ A371) (EFSA, 2004). Expression is under the regulation of the Pna2/TPI promoter, constructed of the neutral amylase II promoter from *A. niger*, where the 5′ non-translated part has been replaced with the 5′ non-translated part of the *Aspergillus nidulans* triose phosphate isomerase (TPI) promoter. In Australia and New Zealand this enzyme preparation is applied as a processing aid for starch (FSANZ A371). Otherwise, it is known as a feed

additive for fattening of monogastric livestock, improving phosphorus utilization in animals fed cereal-based diets (trade name Bio-Feed®).

T. Transglucosidase

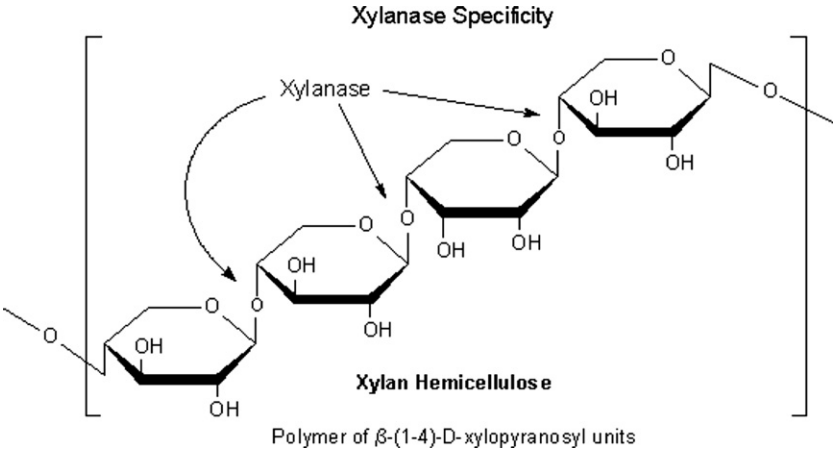
Transglucosidases (EC 2.4.1.24) transfer an α-D-glucosyl residue in a 1,4-α-D-glucan to the primary hydroxy group of glucose, free or combined in a 1,4-α-D-glucan (KEGG).

The transglucosidase gene from *Aspergillus niger* is heterologously expressed in *Trichoderma reesei* strain RL-P37, which has been genetically modified by deletion of several cellulase genes. The expression cassette contains the DNA encoding *A. niger* mature secreted transglucosidase protein fused to the *T. reesei* CBHI signal peptide, for enhanced secretion. This open reading frame is flanked by the promoter and terminator sequences of the *T. reesei* cellobiohydrolase 1 (*cbhl*) gene (FDA-GRN 315).

Transglucosidase from *A. niger* only acts on oligosaccharides with a low degree of polymerization (Goffin *et al.*, 2010). This transglucosidase catalyzes both hydrolysis and transfer of α-D-glucooligosaccharides, resulting in a variety of products (Goffin *et al.*, 2010). Transfer occurs most frequently to HO-6, producing isomaltose from D-glucose, and panose from maltose. Transglucosidase can also transfer to the HO-2 or HO-3 of D-glucose to form kojibiose or nigerose, or back to HO-4 to form maltose. The action on maltose produces an equimolar concentration of panose and glucose. As a result of transglucosidase catalysis, malto-oligosaccharides are converted to isomalto-oligosaccharides containing high proportions of glucosyl residues linked by an α-D-1,6 linkage from the non-reducing end. Therefore, non-fermentable sugars including raffinose and stachyose are converted to sucrose, galactose, glucose, and fructose, which can then be fermented into alcohol (FDA-GRN 315). Therefore, the preparation is used as a processing aid in the production of isomalto-oligosaccharide syrup from starch and potable alcohol from molasses.

U. Xylanase

Xylanases (EC 3.2.1.8) hydrolyze 1,4-β-D-xylosidic linkages in the arabinoxylan backbone:



(Source: sigma.com)

The linear polysaccharide 1,4-β-D-xylan is a component of hemicelluloses, the main constituents of plant cell walls. Arabinoxylans are highly branched xylans that are found in various cereals, and they exist in both a soluble and an insoluble form.

The endo-1,4-β-xylanase gene from *Thermomyces lanuginosus* has been transferred into a selected strain of *Fusarium venenafum* (trade name NOVOZYM® 899) as well as *Aspergillus oryzae* (FDA-GRN 54). The xylanase gene from *T. lanuginosus* is under the control of the *F. oxysporum* trypsin gene promoter and terminator. The derived heterologous xylanase enzyme preparations are used in the food industry as a processing aid in baking. The hydrolysis of the xylosidic linkages in an arabinoxylan backbone results in depolymerization of the arabinoxylan into smaller oligosaccharides. This increases the elasticity of the gluten network, improving handling of the dough.

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