Cry Proteins in Biotechnology

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A hallmark of *Bacillus thuringiensis* bacteria is the formation of one or more parasporal crystal (Cry) proteins during sporulation. The toxicity of these proteins is highly specific to insect larvae, exerting lethal effects in different insect species but not in humans or other mammals. In 1989, a nomenclature was proposed to classify proteins according to their sequence and specificity. In this initial nomenclature, there were only four classes. The first class included proteins with action against Lepidoptera with a size of approximately 130–140 kDa. The second class included smaller proteins (65 kDa) with activity against Lepidoptera and Diptera; this class included only two members: CryIIA and CryIIB. The third class constituted the active toxin against Coleoptera, CryIIIA. The last class was Cry1A, the members of which were closely related: they were called Cry1Aa, Cry1Ab, and Cry1Ac.

Cry proteins adjuvant

1. Introduction

A hallmark of *Bacillus thuringiensis* bacteria is the formation of one or more parasporal crystal (Cry) proteins during sporulation. The toxicity of these proteins is highly specific to insect larvae, exerting lethal effects in different insect species but not in humans or other mammals. *B. thuringiensis* was isolated for the first time in 1902 by the Japanese scientist Ishiwata, who was studying the cause of mortality in silkworm larvae; thus, this disease was also called Soto disease. Ishiwata initially named this bacterium *Bacillus sotto* [1]. A few years later, in 1911, a German scientist, Ernst Berliner, isolated a bacterial strain in dead moth larvae in Mediterranean flour, located in a flour mill in the German state of Thuringia. For this reason, Ernst named this Bacillus *B. thuringiensis* (Bt) [2]. Subsequently, the probable mechanism of cytotoxic action of particular Bt inclusions, called parasporal, was shown in silkworm larvae (*Bombyx mori*). Changes in the permeability of the intestinal walls of the insect were observed, consequently causing its death. These results showed that the parasporal inclusions contained crystals of δ-endotoxin, which was the cause of the larva deaths [3][4]. The successful use of Bt in agriculture lies in the production of crystal proteins called Cry, which have specific cytotoxic activity against different insect orders, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga [5][6][7]. In 1989, a nomenclature was proposed to classify proteins according to their sequence and specificity. In this initial nomenclature, there were only four classes. The first class included proteins with action against Lepidoptera with a size of approximately 130–140 kDa. The second class included smaller proteins (65 kDa) with activity against Lepidoptera and Diptera; this class included only two members: CryIIA and CryIIB. The third class constituted the active toxin against Coleoptera, CryIIIA. The last class was Cry1A, the members of which were closely related: they were called Cry1Aa, Cry1Ab, and Cry1Ac [8]. The activity of Cry proteins as bioinsecticides has been widely studied [9]; however, their other activities, such as their potential adjuvant function, have been inadequately

explored. Although some experiments have confirmed their potential action as immunopotentiators [10][11][12], there are many Cry proteins that have not been evaluated yet. Furthermore, in different studies, the lack of toxicity of these proteins has been demonstrated in mammals, especially humans, and it has even been shown that these proteins can generate an immune response mediated by antibodies [13]. However, it is crucial to determine the mechanism by which Cry proteins are activated to induce an immune response, as well as to evaluate the possible risks that they could pose in the short and long term, such as allergies or other immune alterations.

2. Action Mechanism of Cry as a Bioinsecticide

Crystal proteins have been widely used in genetically modified cultures; these transgenic cultures can produce Bt crystals, making them insect resistant. The two presentations of bioinsecticides, those that contain spores and toxic crystals or transgenic foods that express Cry proteins, have a similar mechanism [14]. Cry protein crystals need to be solubilized for their activation after they are ingested by an insect larva. The activation is mediated by the action of proteolytic enzymes, such as cathepsin G and chymotrypsin, which are located in the digestive system of the insect larva [15] and perform proteolytic processing at the amino terminus of the Cry protein [16]. Once the protein is activated it becomes the so-called δ-endotoxin; this soluble and partially truncated form of the protein is expressed in transgenic foods [14]. The δ-endotoxin binds to receptors located in the membrane of the epithelial cells of the intestine of the larva. Proteins such as cadherins [17], aminopeptidases, and alkaline peptidases [18] are recognized by the domains of δ-endotoxin and, recently, the binding of δ-endotoxin to other proteins, such as ATP-binding transporter proteins (ABC), has been demonstrated [19]. The cadherins that bind to Cry toxins in different orders of insects share a structure composed of four domains: an ectodomain (CE), a domain of the proximal extracellular membrane (MPED), a transmembrane domain (TM), and a cytoplasmic domain (CYTO). Several models have been proposed to explain the action mode of Cry proteins. One of them describes the process in several steps: solubilization of the crystal, processing of the protoxins, and binding to the receptor. The binding allows the oligomerization of δ-endotoxin in the membrane of intestinal cells, its insertion into the membrane, and aggregation, which results in the formation of a pore. This pore in the cell membrane causes an ionic imbalance (release of H^+, K^+, Na^+, and Ca^{2+} ions) in the cell [20][21][22], which causes an increase in cAMP and, consequently, the activation of the apoptotic process known as cytolysis (**Figure 1**) [23]. Another model suggests that the toxin monomer can bind to a cadherin receptor and activate Mg^{2+}-dependent signal transduction, a pathway that leads to cell death [24].

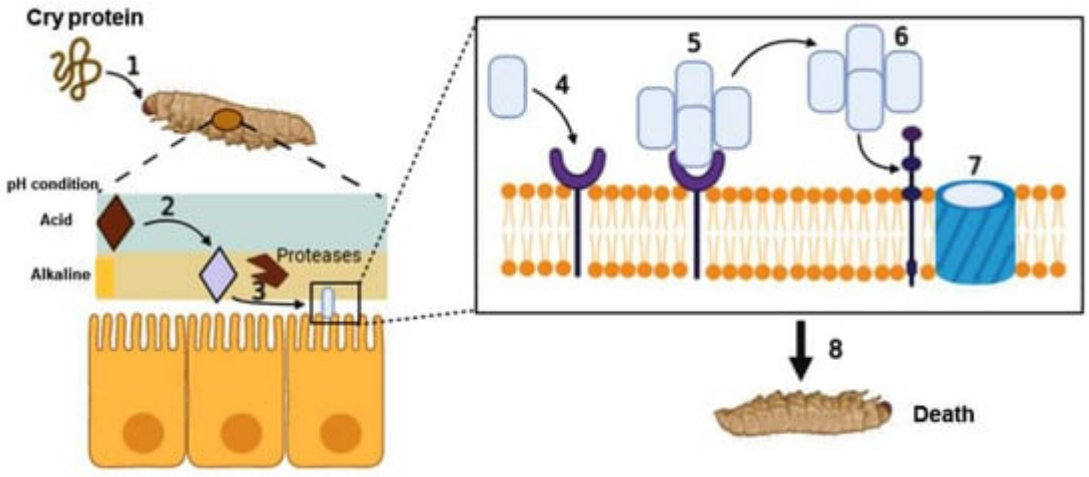

Figure 1. When an insect larva ingests *Bacillus thuringiensis* or the Cry protein present in bioinsecticides, it ingests crystals that may contain one or more Cry proteins (1). These crystals are solubilized due to the alkaline pH present in the midgut of the insect. After that, Cry proteins are released in the form of protoxins (inactive, active), which still lack toxic biological activity. Alkaline pH conditions ranging from 8 to 11 are found in lepidopteran and dipteran insects; some Cry proteins require neutral or slightly acidic pH conditions, which are present in coleopteran insects. Thus, Cry proteins are specific (2). Soluble Cry proteins cannot produce their effects until they are processed by intestinal proteases, generating active toxins, which requires the cleavage of peptides from both the N- and the C-termini (3). Subsequently, they bind to various membrane receptors of the cells of the insect's intestine (4), form oligomers (5) until they locate and bind to a specific receptor, mainly cadherins, among others (see the text) (6), and lead to the formation of a pore (7), causing an osmotic imbalance, cell lysis, and finally, as a consequence, the death of the insect (8).

Some non-target insects and even some mammals, such as humans, are not sensitive to this bioinsecticide despite having the same receptors on the cell membrane; however, a difference in the structures of the receptors has been observed. Cadherin (Type IV) proteins in sensitive insects have eight or more cadherin domains, which facilitate the anchoring of δ-endotoxin, unlike the cadherins of resistant insects, which have few domains. For this reason, δ-endotoxin is specific because it binds to certain receptors in target insects [25][26]. Moreover, the proteins that allow proteolytic processing for the activation of the Cry protein are not present in the digestive system of resistant insects [16].

On the other hand, cadherins (type I) in humans have structural differences compared to insect cadherins; they principally have minor ectodomains (EC) and a few Ca^{2+} insertions, which confer foldability to the consecutive extracellular cadherin domains responsible for homophilic binding. This binding is also different in human cadherins because the EC1 domain of vertebrate cadherins contains a conserved tryptophan residue (W) inserted in the hydrophobic pocket, affecting homophilic binding [27]. Moreover, the identity between the cadherins of humans and those of insects (Diptera, Lepidoptera, and Coleoptera) is very low, ranging from 13% to 20%. For these reasons, Cry proteins do not pose any potential toxicological risk to humans when they are ingested.

3. Cry Proteins

In 1989, a nomenclature was proposed to classify proteins according to their sequence and specificity. In this initial nomenclature, there were only four classes. The first class included proteins with action against Lepidoptera with a size of approximately 130–140 kDa. The second class included smaller proteins (65 kDa) with activity against Lepidoptera and Diptera; this class included only two members: CryIIA and CryIIB. The third class constituted the active toxin against Coleoptera, CryIIIA. The last class was Cry1A, the members of which were closely related: they were called Cry1Aa, Cry1Ab, and Cry1Ac [8].

In 1998, a new nomenclature was published classifying toxins solely by their amino acid sequence. On this basis, most proteins are related and contain up to five conserved domains. Subsequently, a slightly modified name system was adopted in which each toxin receives a name that incorporates four levels. First, in general, toxins that share at least 45% identity in their sequence have the same number. The second rank (A) is used to distinguish sequences that share between 45% and 78% identity. Those that share between 78% and 95% identity are distinguished at the tertiary level (a). Finally, the quaternary range is used to identify certain differences. Subsequently, in 2003, Cry proteins were classified into three groups through a phylogenetic approach. The group with the majority of Cry toxins is known as the family of three domains since they contain three structural domains. This family contains the largest group of Cry proteins, which are globular molecules that contain three structural domains connected by simple bonds. A particular characteristic of the members of this family is the presence of protoxins with two different lengths. Long protoxins are approximately twice the length of most toxins. The C-terminal extension found in long protoxins is dispensable for toxicity and is believed to play a role in the formation of crystal inclusion bodies within the bacteria [8]. To date, the three dimensional structures of 12 Cry toxins without modifications (Cry1Aa6, Cry1Ac7, Cry1Ac8, Cry2Aa, Cry3Aa12, Cry3Aa3, Cry3Bb1, Cry4Aa1, Cry4Ba1, Cry5Ba1, Cry7Ca1, and Cry8Ea1) [28][29][30][31][32][33][34][35][36][37][38], and some with modifications in the form of mutations (Cry1Ac4 and Cry1Da1) [39][40], have been determined by X-ray crystallography (**Figure 2**).

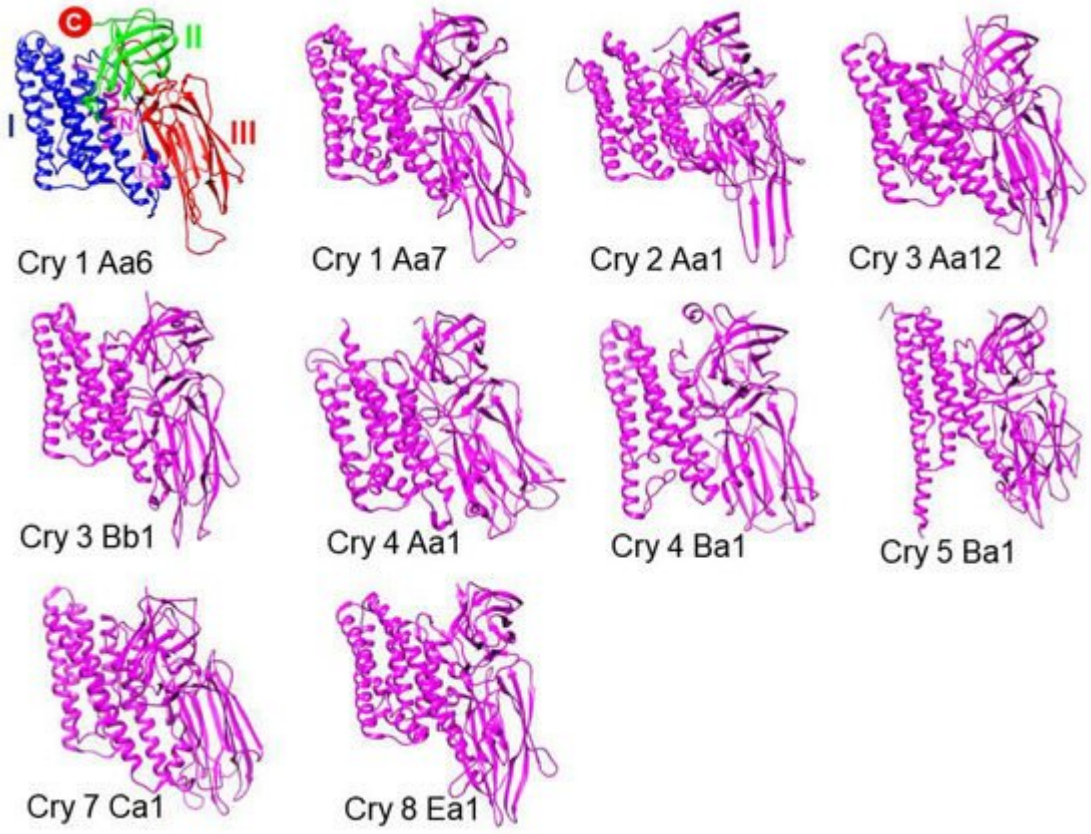

Figure 2. Structural representation of some crystallized Cry proteins (toxins). The Cry 1 Aa6 toxin Domains I-III are indicated in blue, green, and red, respectively, and the N- and C-termini are shown by circles filled with red and white backgrounds, respectively. The Linker connecting Domains I and II is shown by a circle filled with a white background with the letter in magenta. Modeling was performed in UCSF Chimera (https://www.rbvi.ucsf.edu/chimera (accessed on 18 September 2021)).

The tertiary structure of the N-terminal domain, called domain I, is a set of seven α-helices, among which the central α-helix is hydrophobic and surrounded by six amphipathic helices; this helical domain is responsible for membrane insertion and pore formation. Domain II consists of three antiparallel β sheets with exposed loop regions, and domain III is a β sheet [28][29][30][31][32][36]. The most exposed regions in the tertiary structure of the protein are domains II and III, which are involved in receptor binding [40].

The crystal protein consists of proteins called δ-endotoxin. The definition of Cry proteins is any parasporal protein of Bt that shows a toxic effect on an organism, verifiable using bioassays, or any protein that shows similarity to Cry proteins [9].

4. An Overview of Cry as a Natural Adjuvant

A strategy used in the design of vaccines to enhance their immunogenicity includes the co-administration of adjuvants that stimulate and improve immunity. Cry proteins have been described as possible adjuvants for their resistance and stability in highly alkaline environments. The structural characteristics of proteins allow them to

modulate the immune response and function as adjuvants. For this reason, several proteins have been studied in the context of therapeutic proteins [41]. Importantly, a wide variety of proteins have been used as adjuvants to immunize animals intranasally, which can stimulate a protective immune response in the lungs and upper respiratory tract and possibly at distant sites, such as the gastric and genital mucosa [42].

With respect to mucosal vaccines, when rhesus macaques were vaccinated intranasally with a trimeric gp41 protein coupled to virosomes to evaluate an HIV-1 vaccine, IgA antibodies increased in the genital tract, and immunization also prevented transmission of infection [43]. Studies have been conducted to identify proteins able to induce a mucosal immune response, which was initially realized by Guimares et al. when they demonstrated the immunogenicity of the Cry1Ab protein of *Bacillus thuringiensis*. When Cry1Ab was exposed to different pH values, the results showed that the protein was only slightly degraded at pH 2.0 and, most importantly, it maintained its immunoreactivity [44]. Subsequently, Cry proteins were used as adjuvants for their resistance and stability. This was demonstrated by immunizing BALB/c mice intranasally, which is an alkaline environment. After immunization, the mice were protected when they were infected by the bacterium *Streptococcus pneumoniae* [45] or the parasite *Naegleria fowleri* [46].

Because of these characteristics, several research groups have studied the use of Cry as a potential adjuvant [45][46][11][47]. The role of Cry proteins as vaccine adjuvants was initially observed with the Cry1Ac protein, which was administered by different routes, including intragastric, intraperitoneal (i.p.), and intranasal immunization [10]. The last two are the most efficient due to their ability to induce isotypes of IgA and IgG antibodies in the murine model. These antibodies demonstrated protection in animal models when they were infected with the bacterium *Brucella abortus* or with parasites *Naegleria fowleri*, *Plasmodium chabaudi*, *Plasmodium berghei*, and *Taenia crassiceps* [10][46][12][48][49]. In the case of *B. abortus*, an intracellular bacterium, the use of Cry1Ac with the RB51 *B. abortus* strain conferred protection against an intranasal challenge with the virulent strain *B. abortus* 2308 in BALB/c mice. The results showed that the vaccine conferred immunoprotection, as evidenced by a decrease in the splenic bacterial load in immunized animals. The proliferation of cytotoxic TCD8+ cells increased the production of TNF-α and IFN-γ, and the generation of an IgG2a antibody response was also observed. These results indicate that the use of the Cry1Ac protein as a mucosal adjuvant via the intranasal route may be a promising strategy for developing a vaccine against brucellosis [48].

When the Cry1Ac protein was administered together with total extracts of the free-living amoeba *N. fowleri*, the immunized animals had 100% protection against the development of meningoencephalitis; however, when the animals were immunized with the Cry1Ac protein alone, only 60% of infected mice survived [46].

On the other hand, mice previously treated with Cry1Ac and infected with *P. chabaudi* (considered non-lethal) had 100% survival compared to mice previously treated with PBS, which demonstrated 80% survival. Furthermore, mice previously treated with Cry1Ac and subsequently infected with *P. berghei* (lethal parasite) survived longer (12 days) than control mice previously treated with PBS, which died on day 9 post-infection. Regarding the induced immune response, an increase in IFN-γ and TGF-β cytokines was demonstrated, in addition to an increase in the levels of IgG and IgM immunoglobulins, in animals treated and infected with two types of Plasmodium [49].

Conversely, when mice were immunized with the Cry1Ac protein and total lysates of *T. crassiceps*, only 40% protection was observed in the experimentally infected animal model, and mice immunized only with Cry1Ac did not survive [10][46][11][47][12]. These results demonstrate that Cry1Ac alone is not able to generate a specific immune response; however, when it is used in the company of a specific antigen, it potentiates the immune response and can function as an adjuvant. At present, the immunological mechanism underlying the effects of the Cry1Ac protein is not entirely understood, but in macrophages this protein can stimulate the overexpression of surface glycoproteins CD80 and CD86, which stimulate the secretion of TNF-α and IL-6 cytokines, allowing activation of an immune response that promotes the survival of animal models against some experimental infections [50].

Another protein used as a potential adjuvant is Cry1Ab (a protein with 86% homology at the amino acid level with Cry1Ac). When administered intranasally, this protein was not able to generate serum and mucosal IgG antibody responses. It was only able to elicit a low level of IgM and SIgA. In contrast, when Cry1Ab was administered by the intraperitoneal route, it was able to induce high levels of IgG and IgM antibodies, similar to the effects of Cry1Aa and Cry1Ac [51]. In another study, the administration of Cry1Ab (1 µg) to BALB/c mice by the i.p. route induced a mixed Th1-Th2 immune response. No evidence of allergenicity has been observed with the administration of Cry proteins [52], though the levels of leukotrienes, cytokines, and eosinophils have notably increased. However, another study conducted with the direct consumption of Bt corn with Cry1Ab protein showed that the inclusion of Cry1Ab in the diet did not affect the severity of asthma or allergic inflammation induced by ovalbumin [50].

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