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May 28, 2019

Dr. Geoffrey Wong
Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notification of GralNzyme® Glucanase for Use in Poultry Feed by Agrivida, Inc.

Dear Dr. Wong,

Under the Final Rule for the notification of self-determination of "Generally Recognized As Safe" (GRAS) for novel animal feed additives (21 CFR Parts 20, 25, 170 et al., Federal Register, vol. 81, No. 159; August 17, 2016) Agrivida, Inc. is hereby submitting a notification of the conclusion by Agrivida, Inc. that the use of the glucanase enzyme, GralNzyme® AC1 Glucanase, in the feed of poultry is GRAS. This enzyme hydrolyzes β-D-1,4 glucan bonds in soluble nonstarch polysaccharides that are present in certain feed grains, thereby improving the digestibility of these nutrients in animal feeds.

Agrivida's conclusion of the GRAS status of the AC1 Glucanase is based upon scientific procedures and information developed through scientific studies conducted by Agrivida, Inc. and its cooperators. The information upon which this conclusion is based is presented in a document two copies of which are enclosed with this letter. In addition, you will find an electronic file of this GRAS notice in PDF format entitled "AC1 GRASn_24May19.pdf" that is present on the compact disc that accompanies this letter. Copies of each of the scientific reports cited in this document to support the information and conclusions of Agrivida, Inc. are contained within a folder that is also present on the enclosed compact disc.

The complete data and original information that are the basis of this GRAS Notification are available to the Food and Drug Administration for review and copying upon request during normal business hours at our offices located at 78E Olympia Avenue, Medford, MA 01801.

If you have questions or comments related to this notice, please forward them to me.

Sincerely,

James M. Liggon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

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By

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gra^Qzyme.

A thermotolerant β-glucanase feed enzyme expressed in Zea mays

SUMMARY of DATA SUPPORTING a NOTIFICATION of GRAS STATUS for USE in POULTRY FEED

Submitting Company:

Agrivida, Inc.
78E Olympia Avenue
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Submitted by:

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May 24, 2019

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Executive Summary

Agrivida, Inc. has developed a new feed enzyme product to improve carbohydrate utilization in poultry feeds. This enzyme is a glucanase referred to as the AC1 Glucanase in this document, and it will be marketed under the trade name GralNzyme® AC1 Glucanase. The gene encoding the AC1 Glucanase was derived using Gene Site Saturation Mutagenesis (GSSM; Short, 2001) starting from a sequence that had been isolated from an environmental library and that encoded a protein identical to the CelSA endoglucanase of *Thermotoga maritima*. The AC1 gene under the control of a monocot-derived seed-specific promoter was transformed into maize (*Zea mays*) using *Agrobacterium*-mediated plant transformation techniques. The resulting transformed maize produces 150 to 300 units of glucanase activity per gram of grain.

The AC1 product is produced using common agronomic practices for the production of maize grain, followed by milling to form a coarse meal. The coarse meal will be added as a feed additive at relatively low inclusion levels to the feed of poultry (200 to 500 units per kg of feed). The intended effect of the AC1 enzyme in animal feed is to improve the digestibility of feed in the animal's gastrointestinal tract through the solubilization of non-starch polysaccharides (NSP) in the diet, thereby reducing the viscosity of the digesta and improving access of the animal's digestive enzymes to nutrients in the diet.

Agrivida, Inc. has conducted and published studies and reports that demonstrate the AC1 Glucanase product's safety and efficacy and support a conclusion that the AC1 Glucanase product is generally recognized as safe (GRAS) for its intended use. The details and results of the studies and reports that support the functionality and a conclusion of the GRAS nature of the AC1 Glucanase are presented herein.

The AC1 gene construct that was used in the transformation of maize contained one copy of the AC1 gene, under the control of the *Oryza sativa*-derived glutelin-1 seed specific promoter. The maize AC1 gene transformants were selected using the well-known phosphomannose isomerase (*pmi*) gene whose safety and utility has been well-established.

The maize event that produces the AC1 Glucanase contains a single T-DNA insertion in its genome. The AC1 gene insertion is located on maize chromosome (b)(4) and it contains the complete transfer DNA (T-DNA) with one copy of the AC1 gene. The complete sequence of the insertion including approximately 1.5 kilobase pairs (Kb) of flanking maize DNA on each side of the insert was determined. The plasmid that contains the T-DNA fragment that was used to transform maize contains an antibiotic resistance gene for maintenance in bacterial hosts. The antibiotic resistance gene was not transformed into the maize genome since it is not located within the T-DNA region of the AC1 gene transformation plasmid. The absence in the maize genome of the antibiotic resistance gene and other elements of the transformation plasmid outside of the T-DNA was confirmed by Southern

hybridization techniques. The stability of the AC1 gene insertion in maize over multiple generations was also demonstrated.

The AC1 Glucanase enzyme derived from three representative product batches was fully characterized. The molecular weight, immunoreactivity and glucanase activity were confirmed. The pH and thermal tolerance profile for the AC1 Glucanase were determined and the N-terminal amino acid sequence of the AC1 Glucanase was confirmed.

It was further demonstrated that the AC1 Glucanase produced by maize is not glycosylated and, consistent with other enzymes in the Glycohydrolase Family 5 group, also possesses endo-cellulase, exo-cellulase and endo-mannanase activities. Three AC1 product batches were demonstrated to meet all JECFA specifications for food enzymes with the exception of number of coliforms and total bacteria. However, the product is within the range for coliforms and total bacteria that are known to be typical for maize grain that is produced by common agricultural practices and widely used in food and feed.

The functionality of the AC1 Glucanase in poultry was demonstrated through viscosity measurements of the digesta in the ileum of live broilers in two broiler chicken feeding studies (Ayres *et al.*, 2018) and in one *in vitro* feed viscosity study (Ayres *et al.*, 2019). In addition, a chicken performance study that included chickens fed diets treated with the AC1 Glucanase is presented that support the functionality of the product in poultry feed (Ayres *et al.*, 2018; Jasek *et al.*, 2018).

Viscosities of digesta from chickens consuming a basal diet high in soluble NSPs (i.e. wheat supplementation) that was supplemented with different amounts of AC1 Glucanase were compared to: a negative control group (NC) that was fed the same basal diet without AC1 Glucanase supplementation, a positive control group (PC_1) fed a conventional corn/soybean diet with higher energy than the NC diet, and to a second positive control group (PC_2) fed the NC basal diet with a higher energy level that was identical to PC_1. AC1 consistently reduced the viscosity of the digesta *in vivo*. The results of one of these studies were reported by Ayres *et al.* (2018). This result is consistent with *in vitro* studies which show AC1 reducing the viscosity of feed mixtures (Ayres *et al.*, 2019).

The AC1 Glucanase product is concluded to be safe based upon the history of safe use of glucanase enzymes in animal feed and the safety of the maize production host. In addition, a high dose of the AC1 Glucanase (5000 U/kg feed) was included in one of the chicken feeding studies to assess the safety of high doses of AC1 in chickens. Assessments of key hematological measurements of the high AC1 dose groups were compared to those of the negative control (NC) and positive control (PC) groups and there were no indications of toxicity or abnormalities in the high AC1 dose groups. Further, post-mortem examinations of animals from the high AC1 dose groups did not reveal any indications of abnormalities or toxicity. The results of this study were published by Broomhead *et al.* (2019).

Based on the above information which is supported by the information contained in this document, Agrivida, Inc. concludes that the AC1 Glucanase product is safe and effective, and is GRAS when used as intended in the feed of poultry.

Introduction

Cereal grains are broadly classified into two major categories, viscous and non-viscous cereals, depending on their content of soluble non-starch polysaccharides (NSPs). Rye, barley, oats, and wheat contain considerable amounts of soluble NSP and are classified as viscous grains, whereas corn, sorghum, millet and rice contain reduced amounts of soluble NSP and are considered to be non-viscous cereals. Due to the high content of viscous NSP in grain of the former, feeds produced from these grains result in high digesta viscosity in the gastrointestinal (GI) tracts of monogastric animals resulting in reduced nutrient digestibility and availability, negative impacts on the gut microbiome and other negative effects (Burnett, 1966; Choct and Annison, 1992; Bedford and Classen, 1992; Danicke *et al.*, 1999). Since the 1980s glucanase and other enzymes that degrade soluble NSP have been added to the feed of monogastric animals to increase performance of animals fed diets based on grains with a high soluble NSP content (Hesselman and Åman, 1986; Campbell *et al.*, 1989; Broz and Frigg, 1986; Newman and Newman, 1987). A large number of enzymes categorized as NSPase are approved for use in animal feed to depolymerize soluble NSPs and improve the digestion of nutrients in feeds based on grains high in soluble NSP content. These include glucanase as well as galactosidase, mannanase, pectinase, and xylanase. β-glucans are a primary soluble NSP of barley, wheat and other grains high in soluble NSPs and are present at levels of 0.2-0.7% in wheat and 1.9-5.4% in barley (Havrlentová and Kraic, 2006). β-glucan is a glucose polymer containing a mixture of β-1-3 and β-1-4 linkages that make its physicochemical properties different from cellulose that is a straight-chain glucose polymer with only β-1-4 linkages. Four types of endo-acting glucanases, classified according to the type of glycosidic linkage they cleave, are capable of depolymerizing (1,3)-(1,4)-β-D-glucan: endo-(1,3)-(1,4)-β-glucanases, endo-1,3(4)-β-glucanases, endo-1,4-β-glucanases, and to a lesser extent, endo-1,3-β-glucanases (McCarthy *et al.* 2003).

Although glucanases have been widely used in feeds based on grains high in soluble NSPs, their utility in corn-soybean meal based diets has also been demonstrated. NSPs in corn-soybean meal based diets have been shown to decrease the digestibility of nutrients by restricting access of digestive enzymes such as amylase and proteases to nutrients intertwined in fibrous cellular matrices (Cowieson, 2005). In addition, legume (e.g., soybean) NSPs are more complex in structure than those of cereals, containing a mixture of colloidal polysaccharides (galacturonans, galactan and arabinans). Accordingly, the addition of pectinase to a corn-soybean meal diet has been shown to significantly increase the metabolizable energy (ME) value of the diet. This improvement in the ME coincided with increased digestibility of galactose-rich polysaccharides (Kocher *et al.*, 2002).

Agrivida, Inc. is developing animal feed enzyme products that are produced in maize grain. Genes encoding the enzymes, under the regulation of monocot-derived seed-specific promoters, are transformed into maize. The enzyme products produced in this manner will be marketed under the trade name of GraINzyme®. One of the

GralNzyme® products under development by Agrivida, Inc. is a glucanase feed enzyme whose primary activity is endo-1,4-β-glucanase but that also exhibits lesser levels of other NSPase activities, including endocellulase, exocellulase, and endo-mannanase. The AC1 Glucanase was developed using GSSM, resulting in the introduction of 12 amino acid changes into the coding sequence of the Cel5A glucanase sequence (NCBI accession Q9X273) (Nelson *et al*, 1999) to improve the thermostability of the AC1 Glucanase protein. The AC1 Glucanase consists of 316 amino acids that share 96% identity with the Cel5A cellulase from *Thermotoga maritima* GH5.

Maize plants engineered to express this gene using Agrivida's GralNzyme® technology produce 150 to 300 units of glucanase activity/g of grain. The GralNzyme® Glucanase product referred to herein as AC1 Glucanase will consist of coarsely ground corn meal produced from maize plants expressing the glucanase gene in the grain. It will be included in relatively small amounts as a feed additive in poultry diets in order to reduce the viscosity of digesta and improve the digestibility of major feed ingredients.

1.0 Signed statements and certification

1.1. Submission of a GRAS notice

Agrivida, Inc. is hereby submitting a GRAS notice in accordance with §170.225(c) of 21 CFR Parts 20, 25, 170 and 570 *et al* (Federal Register, Vol. 81, No. 159, August 17, 2016) for an endo-1,4-β-glucanase enzyme for use in the feed of poultry to improve the digestibility of feeds containing soluble non-starch polysaccharides (NSP).

1.2. Name and address of notifier

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801 USA
Tel: 781-391-1262

Person responsible for the dossier:

James Ligon, PhD
Agrivida, Inc.
VP, Regulatory Affairs and Stewardship
1023 Christopher Drive
Chapel Hill, NC 27517 USA
Tel: 919-675-6666
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1.3 Name of the notified substance

The substance that is the subject of this GRAS notice is an endo-1,4-β-glucanase enzyme with high similarity to the *Thermotoga maritima* CelSA glucanase enzyme (E.C. 3.2.1.6). The glucanase is produced in the grain of *Zea mays*. The trade name of the product is GralNzyme® AC1 Glucanase.

1.4 Conditions of use of the notified substance

This GRAS notice is for the purpose of establishing GRAS status for GralNzyme® AC1 Glucanase in the feed of poultry in order to increase the digestibility of feed containing soluble NSP. The recommended inclusion rate of the GralNzyme® AC1 Glucanase in poultry feed is 200 to 500 glucanase activity units (U) per kg of feed.

1.5 Statutory basis for conclusion of GRAS status

The conclusion that the GralNzyme® AC1 Glucanase enzyme is GRAS for use in poultry feeds is based on scientific procedures in accordance with 21 CFR Part 570, Subpart E (Federal Register, Vol. 81, No. 159, August 16, 2016).

1.6 Substance is exempt from premarket approval

It is the opinion of Agrivida, Inc. that the GralNzyme® AC1 Glucanase is exempt from the requirement for premarket approval under the Food, Drug and Cosmetic Act based on our conclusion that it is GRAS for its intended use in the feed of poultry.

1.7 Data availability

The data that are the basis for the conclusion that the GralNzyme® AC1 Glucanase is GRAS for its intended use are fully encompassed by this submission, including published manuscripts and reports. In addition, upon request by the FDA, Agrivida, Inc. will produce any additional relevant information either in paper or suitable electronic form and provide these to the FDA.

1.8 Confidential business information in this GRAS notice

Agrivida, Inc. does not consider information contained in this document to be confidential business information.

1.9 Certification

Agrivida, Inc. hereby certifies that to the best of its knowledge, this GRAS notice includes all relevant information, both favorable and unfavorable, that is pertinent to the safety and functionality of the GralNzyme® AC1 Glucanase for its use in the feed of poultry.

1.10 Signatory person

Date: May 24, 2019

James M. Ligon, Ph.D.
Vice President, Regulatory Affairs and Stewardship
Agrivida, Inc.

1.11 Authorization to send trade secrets

If necessary, Agrivida, Inc. authorizes FDA Center for Veterinary Medicine to send information from this notification, including information considered by Agrivida, Inc. to be trade secret or CBI, to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture.

2.0 Identity, method of manufacture, specifications and technical effect

2.1 Identification of the notified substance

The GralNzyme® AC1 Glucanase product developed by Agrivida, Inc. is an endo-1,4-β-glucanase (EC 3.2.1.6) produced by genetically modified *Zea mays* (corn).

The gene encoding the AC1 Glucanase was derived from a gene isolated from an environmental DNA library that is identical to the CelSA glucanase gene of *Thermotoga maritima* (NCBI accession Q9X273) (Nelson *et al.*, 1999). The gene sequence was modified by GSSM to improve its thermostability. The resulting AC1 glucanase differs from the CelSA glucanase by (b) (4) amino acids. Expression of the AC1 gene is directed by a monocot-derived seed-specific promoter, such that the GralNzyme® AC1 Glucanase is produced only in the grain of *Z. mays*. Detailed information about the production organism, enzyme, manufacturing process and safety of the GralNzyme® AC1 Glucanase for use in poultry nutrition is contained in this submission. The production host organism, *Z. mays*, is well-characterized with respect to safety and toxicology and is considered safe for consumption by humans and animals.

2.2 Method of manufacture

The GralNzyme® AC1 Glucanase product is produced by cultivating *Z. mays* producing GralNzyme® AC1 Glucanase using common agricultural procedures for producing maize grain, and the harvested grain containing GralNzyme® AC1 Glucanase is milled to a coarse meal and packaged in suitably labeled containers for inclusion in poultry feed.

3.0 Target animal exposure and safety factor calculation

The AC1 Glucanase product will be marketed according to its label specifications. The product label will provide instructions for the user to include the product in the feed of poultry at a rate of 200 – 500 U/kg. Therefore, the highest dose in this range equal to 500 U/kg was used to estimate dietary exposure. It is assumed that the users of this product will follow the product dose recommendations provided by Agrivida, Inc. on the product label and so no other significant exposure to animals other than poultry consuming feed treated with the AC1 Glucanase is expected.

Exposure to other substances as a result of the use of this product is primarily limited to the breakdown products of β-1,4 glucans as would be expected with the use of any other glucanase product or the normal digestion of β-1,4 glucans. Since the AC1 Glucanase also exhibits lesser amounts of endocellulase, exocellulase, and endo-mannanase activity, the products from these activities, including β–glucose, oligosaccharides and polysaccharides, will also be released from the diet. However, all of these products are nutritious and are normally released from complex carbohydrates in the diet during normal digestion so the level of exposure to these is not expected to be substantially different.

Since the AC1 Glucanase is contained within the grain of maize and the nutritional composition of the grain is not different from that of conventional maize grain, the only exposure to other products would be those derived from maize grain that are considered safe for food.

4.0 Self-limiting levels of use

The GralNzyme® AC1 Glucanase product is not intended for inclusion in human food and it will be marketed with a label that states that the product is to be used only for inclusion in poultry feeds. Therefore, according to §170.240 of 21 CFR Parts 20, 25, 170 *et al* (Federal Register, Vol. 81, No. 159, August 16, 2016) there is no requirement to establish a self-limiting level of use for the GralNzyme® AC1 Glucanase product.

The GralNzyme® AC1 Glucanase is produced by maize genetically engineered with a glucanase gene that is closely related to the *CelSA* cellulase gene of *Thermotoga maritima*, and produces GralNzyme® AC1 Glucanase in the grain. Typically, grain derived from the maize production host contains between 150-300U per gram of grain. Other than the presence of the GralNzyme® AC1 Glucanase enzyme, the GralNzyme® AC1 Glucanase product contains maize grain that is nutritionally equivalent to normal maize grain used as a major poultry feed ingredient. The presence of the GralNzyme® AC1 Glucanase in maize grain does not appear to affect the taste, palatability or other organoleptic properties of the grain. Therefore, the maximum amount of GralNzyme® AC1 Glucanase product that might be theoretically consumed by an animal is equal to the total amount of maize meal included in the feed. In the case of poultry feed based on a maize/soybean meal diet, the maize component typically comprises between 50% and 70% of the total feed. Accordingly, the maximum amount of GralNzyme® AC1 Glucanase that might be consumed by poultry is equivalent to the amount of GralNzyme® AC1 Glucanase contained in the maize component of the diet assuming that all of the maize meal was GralNzyme® AC1 Glucanase product. However, since the GralNzyme® AC1 Glucanase product will be marketed primarily in either 20 kg bags or 1 ton totes with a product label that directs the user to add the appropriate amount of the product when mixing the feed, the likelihood that a feed would be prepared using the GralNzyme® AC1 Glucanase product to replace all of the maize meal in the diet is very remote.

Nevertheless, assuming that a 1 ton tote of GralNzyme® AC1 Glucanase product was used in place of normal maize meal to make a poultry feed, the maximum amount of feed that could be produced would be 2 tons or less. In the unlikely event that this transpired, the resulting feed would not be expected to cause adverse effects on the poultry that consume it, even at a maximum dose of approximately 210,000 U/kg as potentially could be expected. Glucanase is an enzyme whose primary enzymatic activity is the depolymerization of 1,3-1,4-β-D-glucan. If large amounts of glucanase were included in a feed it would be expected that most or all of the 1,4-β-D-glucan bonds in the diet would be hydrolyzed to produce simpler carbohydrates with resulting increased levels of energy availability.

Based on the above, it is expected that in the unlikely event that grain from GralNzyme® AC1 Glucanase expressing maize were to be substituted for all of the maize in a typical maize/soybean meal poultry diet that it would not adversely

affect the performance of the poultry nor would it cause any safety concerns for the animals. Additionally, the AC1 Glucanase protein would not be expected to be present in the meat derived from animals consuming it since it has been well established that ingested proteins are digested and absorbed as small peptides and amino acids (Metcalf *et al.*, 1996; Betz *et al.*, 2000). Therefore, the meat of animals consuming feed treated with GraNZyme® AC1 Glucanase protein would be safe for human consumption.

5.0 Experience based on common use prior to 1958

The GraINzyme® AC1 Glucanase product was not in use prior to 1958 and Agrivida, Inc.'s conclusion of GRAS status for the use of this product in poultry feed is not based on its common use prior to 1958. Agrivida's conclusion that the GraINzyme® AC1 Glucanase product is GRAS for use in poultry feed is based on scientific principles. Therefore, the requirement to provide evidence of its use prior to 1958 is not applicable.

6.0 Narrative

6.1 Safety of GralNzyme® AC1 Glucanase

The characterization and safety assessment of feed enzymes produced through the use of recombinant DNA technology involves a number of factors that include the following:

1) The safety of the organism that was the source of the gene encoding the enzyme
2) The safety of the host/recipient organism, in this case *Zea mays*
3) Characterization of the construct used to transform the host/recipient
4) Characterization of the production organism i.e. the transgenic *Zea mays* used to produce the enzyme
5) The characterization and safety of the enzyme itself.

Each of the above factors in relation to the safety of the AC1 Glucanase is discussed in this section.

6.1.1 Source of the gene encoding AC1 Glucanase

The AC1 gene encoding the GralNzyme® AC1 Glucanase enzyme was derived from a gene isolated from an environmental DNA library. The isolated gene encoded an enzyme that is identical to the CelSA cellulase from *Thermotoga maritima* (NCBI accession Q9X273) (Nelson *et al.*, 1999). This gene was modified by GSSM resulting in (b) (4) amino acids changes in order to improve the thermostability of the AC1 Glucanase. The modified gene encodes a glucanase enzyme that is a 37.7 kDa protein with 96% identity to the CelSA glucanase of *T. maritima*. Agrivida, Inc. has compared the CelSA and AC1 glucanase enzymes and has demonstrated that the enzyme kinetics of these two glucanases is nearly identical (Table 1). Expression of the AC1 gene is directed by a monocot-derived seed-specific promoter, such that the GralNzyme® AC1 Glucanase is produced only in the grain of *Z. mays*. Detailed information about the production organism, enzyme, manufacturing process and safety of the GralNzyme® AC1 Glucanase for use in poultry nutrition is contained in this submission. The production host organism, *Z. mays*, is well characterized with respect to safety and toxicology and is considered safe for consumption by humans and animals.

Table 1. Comparison of the enzyme kinetic properties of the CelSA glucanase of *T. maritima* and the AC1 Glucanase produced by maize Event FG259.

	Vmax (umoles/min/mg	Kcat min^{-1}	Km mg/mL
CelSA	36.5	1365	0.38
AC1	31.2	1167	0.22

Since only the coding sequence of the *AC1* Glucanase gene and no other DNA derived from the original host is included in the transformation construct of plasmid (b) (4), the identity of the source organism or its safety profile is not relevant to a discussion of the safety of the AC1 Glucanase protein.

6.1.2 Description of the Host Organism

Taxonomy of *Zea mays* L. (Maize)
The taxonomy of maize is described by OECD (2003) as follows:
Family: Poaceae
Subfamily: Panicoideae
Tribe: Maydeae
Genus *Zea*[1]
Section Zea
Zea mays L. (maize)
Zea mays subsp. *mays* (L.) Iltis (maize, 2n[2] =20)
Zea mays subsp. *mexicana* (Schrader) Iltis (teosinte, 2n = 20))
race Nobogame[3]
race Central Plateau[3]
race Durango[4]
race Chalco[3]
Zea mays subsp. *parviglumis* Iltis and Doebley (teosinte, 2n = 20)
var. *parviglumis* Iltis and Doebley (=race Balsas)
var. *huehuetenangensis* Doebley (=race Huehuetenango)

[1]Iltis and Doebley, 1980; Doebley *et al.*, 1990. [2]Diploidy number. [3]Wilkes, 1967.
[4]Sánchez-González *et al*, 1998.

Maize (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads (Hitchcock and Chase, 1971). Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Maize is wind-pollinated and both self and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes but can remain viable for longer durations under favorable conditions (Coe *et al.*, 1988). Cultivated maize is presumed to have been derived from teosinte (Z *mexicana*) and is thought to have been introduced into the Old World in the sixteenth century. Maize is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No native toxins are reported to be associated with the genus *Zea* (International Food Biotechnology Council, 1990).

As discussed above, the indigenous peoples of North America have cultivated maize for thousands of years. The modern era of maize hybrid production began in the United States where research conducted in the early part of the 20th century proved that hybrid maize could produce a yield superior to open-pollinated varieties (Sprague and Eberhart, 1976). Gradually, hybrid-derived varieties replaced the open-pollinated types in the 1930's and

1940's. Almost all maize grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises, and the older open-pollinated varieties are virtually unknown in commerce.

Maize is planted when soil temperatures are warm (greater than or equal to 10°C) usually mid to late April until mid-May in the U.S. Corn Belt. Optimum yields occur when the appropriate hybrid maturity and population density are chosen. In addition, exogenous sources of nitrogen fertilizer are generally applied and weed and insect control measures are generally recommended. Choice of the appropriate hybrid for the intended growing area helps to ensure that the crop will mature before frost halts the growth of the plant at the end of the season; hybrids are categorized according to the amount of Growing Degree Units (GDU) that will be required for maturity (Monsanto, 2015). Therefore, a hybrid developed for a specific climate zone will not mature in cooler areas that receive fewer GDUs during a typical growing season.

Maize is the largest cultivated crop in the world and is widely cultivated in most areas of the world. In 2014 the global production of maize grain was 1,275 million metric tons (MT), including the 381 million MT produced in the U.S. from planting over 90 million acres (USDA FAS, 2014). In the U.S., maize is grown in almost every state.

In 2015, there were about 88 million acres planted to maize in the United States that produced 13.6 billion bushels of grain and 128 million tons of silage (USDA-NASS, 2017). Maize grown in the United States is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed by wet or dry milling to yield products such as high fructose corn syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, maize starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds.

Conventional plant breeding results in desirable characteristics in a plant through the unique combination of genes already present in the plant. However, there is a limit to genetic diversity with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of highly desirable traits that are beneficial to growers and animal producers.

Given the long history of the safe use of maize grain and its by-products and maize silage as food and feed ingredients, maize and its grain are considered to be generally recognized as safe (GRAS). Therefore, it is concluded that maize and grain produced by it are safe for consumption by humans and animals and that its cultivation does not present any threats to the environment. Pariza and Foster (1983) developed a decision tree to determine the safety of food and feed enzyme preparations that was updated by Pariza and Johnson (2001) and Pariza and Cook (2010). A key tenet of this decision tree is that since enzymes by themselves are not toxic, the primary consideration of the safety of a food enzyme

preparation is the safety of the production organism. In the case where the production organism is a plant that has a long history of safe use as a food ingredient, the enzyme preparation from such a plant is considered to be safe and nontoxic. Based on the decision tree for establishing the safety of food enzyme preparations by Pariza and coauthors (Pariza and Foster, 1983; Pariza and Johnson; 2001; Pariza and Cook, 2010) and on the established long history of safe use of maize for food and feed, the AC1 enzyme preparation that is the subject of this document is considered to be safe for its intended use in poultry feed.

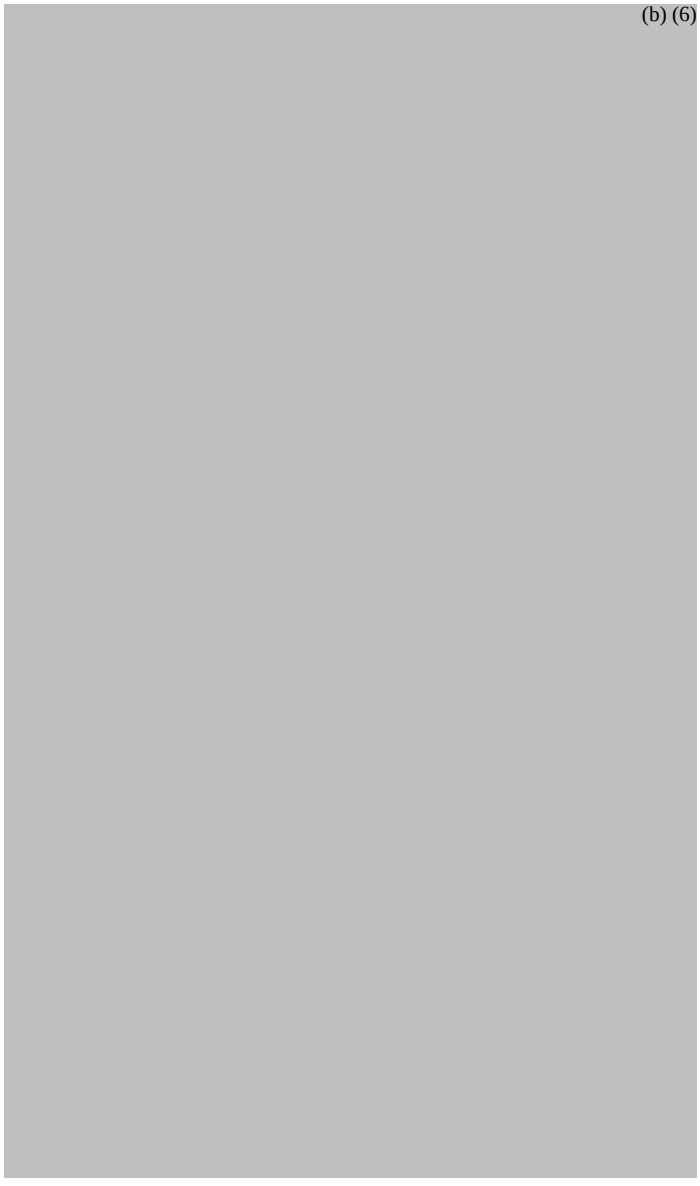
6.1.3 Source of the maize line

The AC1 gene responsible for the production of AC1 Glucanase in maize was initially transformed into a maize line named (b) (4) maintained by the U.S. National Plant Germplasm System (MGSC, 2018) that is also known by the name (b) (4) (herein abbreviated as "B"). The resulting transgenic plants (T0) containing the AC1 gene were subsequently crossed with male and female maize inbreds, (herein abbreviated as "E" and "G" respectively). Several additional backcrosses (BC) with the AC1 gene progeny were performed using the E and G inbred lines in order to increase the percentage of the genome from these inbred lines in the AC1-producing derivative lines. A breeding diagram showing the recent breeding activity for the development of AC1 Glucanase- producing maize is shown in Figure 1.

In addition to the above, the Organization for Economic Co-operation and Development (OECD) Consensus Document on the Biology of Maize (OECD, 2003) provides key information on:

- the biology of maize, including taxonomy and morphology and use of maize as a crop plant
- agronomic practices in maize cultivation, geographic centers of origin, reproductive biology, and cultivated maize as a volunteer weed
- inter-species/genus introgression into relatives and interactions with other organisms
- a summary of the ecology of maize.

Figure 1. A breeding diagram for the development of Event FG259 maize lines described in this document



6.1.4 Characteristics of the AC1 Glucanase gene expression construct

A transformation gene cassette containing a gene encoding the AC1 Glucanase under the control of the (b) (4) (b) (4) (b) (4) (b) (4) signal was constructed in plasmid (Figure 2). The genetic elements of the T-DNA fragment that was used to transform maize are described in Table 2 and presented in Figure 2. This plasmid was transformed by *Agrobacterium*-mediated transformation into immature corn embryo tissue as described by Negrotto et al. (2000) and transformants were selected based on the presence of the plant selectable marker

phosphomannose isomerase (PMI) gene (*manA*) on the transformed DNA fragment that encodes the PMI enzyme. PMI enables corn tissue to grow on culture media containing mannose as the primary carbon source (Negrotto *et al.*, 2000). The *manA* gene has been used as a selectable gene in several genetically modified corn varieties that have completed review by the USDA, FDA, and EPA for food and feed safety, including corn events (b) (4) and (b) (4) corn with resistance to corn rootworm, lepidoptera-resistant (b) (4), and α-amylase expressing event 3272, all products of (b) (4). Corn plants containing the AC1 Glucanase gene were cultivated and produced grain accumulating 150 to 300 units of endoglucanase activity per gram of grain.

In addition to containing the AC1 Glucanase coding sequence, (b) (4) . Agrivida, Inc. has conducted N-terminal amino acid sequencing of AC1 (b) (4) The transformation event chosen for commercial development was designated maize Event FG259.

Figure 2. Genetic map of (b) (4)**, the plant transformation vector containing the AC1 Glucanase gene. The genetic elements of this vector are described in Table 2. Locations of the cleavage sites for restriction enzymes *Bam*HI, *Eco*RI, *Sph*I and *Hind*III are shown**

Table 2. Description of the genetic elements in the (b) (4) bp vector plasmid (b) (4) containing the AC1 Glucanase gene.

(b) (4)					

Figure 3. Comparison of the deduced amino acid sequences of the AC1 Glucanase and the *Thermotoga maritima* CelSA glucanase. Amino acid differences in the AC1 Glucanase relative to the CelSA glucanase are shaded in green. Identical amino acid residues in the two proteins are indicated in the third row as asterisks (*), conservative replacements as colons (:), and non-conservative replacements as spaces.

(b) (4)

(b) (4)

(b) (4)

6.1.5 Characterization of Maize Event FG259

6.1.5.1 Determination of number of DNA insertions

Southern hybridization was used to determine the number of T-DNA insertions from transformation plasmid (b) (4) in the genome of Event FG259. Genomic DNA from Event FG259 was digested with the restriction endonuclease *BamHI* as well as with *HindIII* and *SphI* combined. Each of these enzyme cuts within the T-DNA and/or in the genomic flanking DNA, resulting in one unique DNA restriction fragment for each locus that contains the *manA* selectable marker gene. The unique DNA restriction fragment can be visualized on a Southern blot by hybridization with a labelled DNA probe that contains the *manA* gene (probe D, Figure 4b). The number of unique DNA fragments that hybridize to the *manA* gene probe indicates the number of T-DNA insertions in the maize genome. As a positive control (b) (4) vector DNA and, as a negative control wild type maize DNA, were digested with *EcoRI* and included on the blot. The results demonstrate that in both BamHI and HindIII/SphI digested genomic DNA of Event FG259 a single DNA fragment was visualized on the Southern blot shown in Figure 5, thereby demonstrating the presence of a single genomic T-DNA insertion in the genome of Event FG259. The *manA* gene probe hybridized as expected to the (b) (4) DNA but failed to hybridize to DNA from wild-type maize that is not transformed with the AC1 gene cassette. These results confirm that there is a single T-DNA insertion in the genome of Event FG259 and they are summarized in Table 3.

Figure 4. (a) Diagram of the locus of the AC1 gene T-DNA insertion within chromosome (b) (4) of the maize genome, (b) Diagram of the transformation vector (b) (4), showing regions from which probes (red bars) were derived for Southern blots. Cleavage sites for key restriction enzymes are shown. The labels of other genetic elements are as listed in Table 2.

(b) (4)

Figure 5. Southern blot of restriction endonuclease-digested FG259 DNA probed with a PMI gene containing DNA probe (Probe D in Figure 4b).

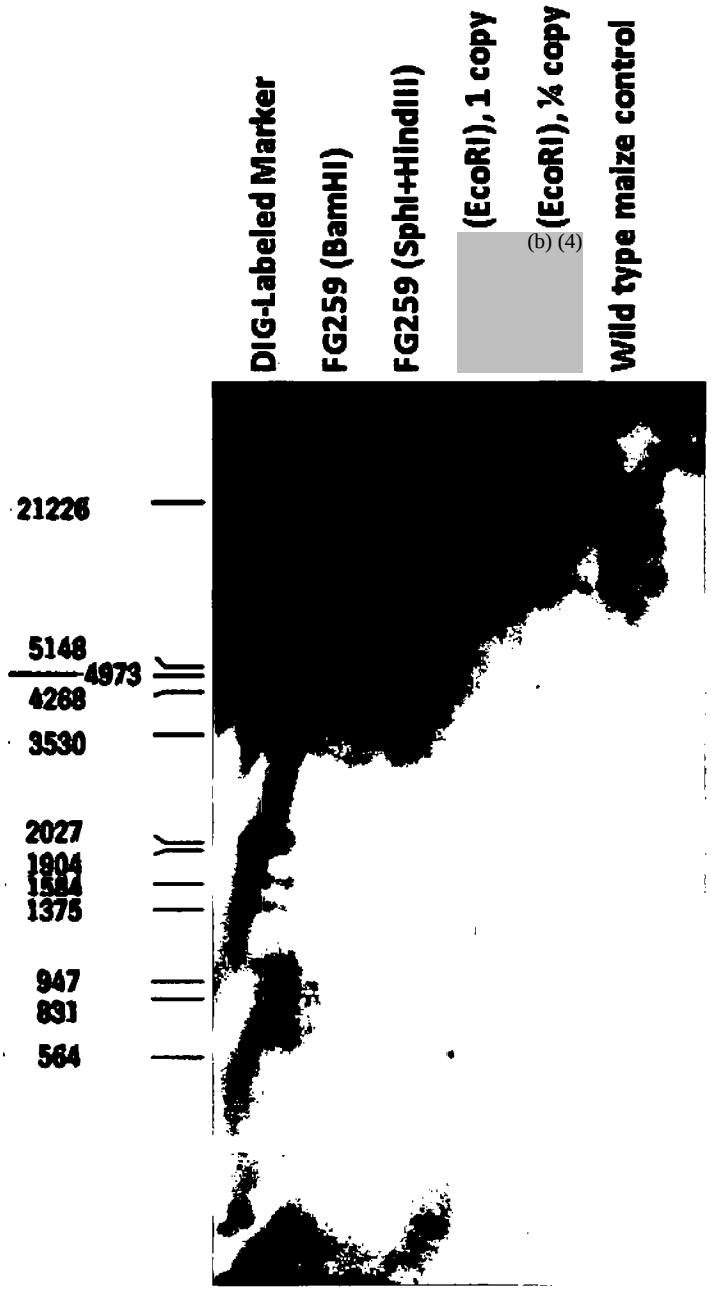


Table 3. Predicted and observed DNA fragment sizes for a Southern blot with the PMI gene probe [Probe D, Figure 4b).

Probe	Sample	Predicted Fragment Size	Observed Fragment Size
T-DNA PMI+	FG259 (BamHI)	6,195 bp	≈6,000 bp
T-DNA PMI+	FG259 [SphI+HindIII)	8,733 bp	≈9,000 bp
T-DNA PMI+	(b) (4) (EcoRI), 1 & ¼ copy	8,748 bp	≈9,000 bp
T-DNA PMI+	Wild type maize control	"	"

Additional Southern blots were used to confirm the above-described findings that Event FG259 contains a single T-DNA insertion. Genomic DNA from five individual Event FG259 plants was pooled and digested independently with restriction enzymes *BamHI* and *HindIII*. While *BamHI* cuts once in the T-DNA, *HindIII* does not, but both enzymes cut in the flanking genomic DNA [Figure 4a). Replicate Southern blots were hybridized with four probes corresponding to T-DNA regions including the GTL promoter [Probe A), the AC1 Glucanase gene [Probe B), the ZmUbi1 promoter [Probe C) and the PMI *manA* marker gene [Probe D) [Figure 4b). Wild type non-transgenic [WT) maize DNA was digested with the same restriction enzymes and run side by side with restriction digests of Event FG259 DNA as a control. The Southern blot probed with T-DNA probes A, B and D is presented in Figure 6, and it shows a single hybridization band in FG259 DNA digested with *BamHI* and *HindIII*, respectively, while no hybridization was found in WT DNA control.

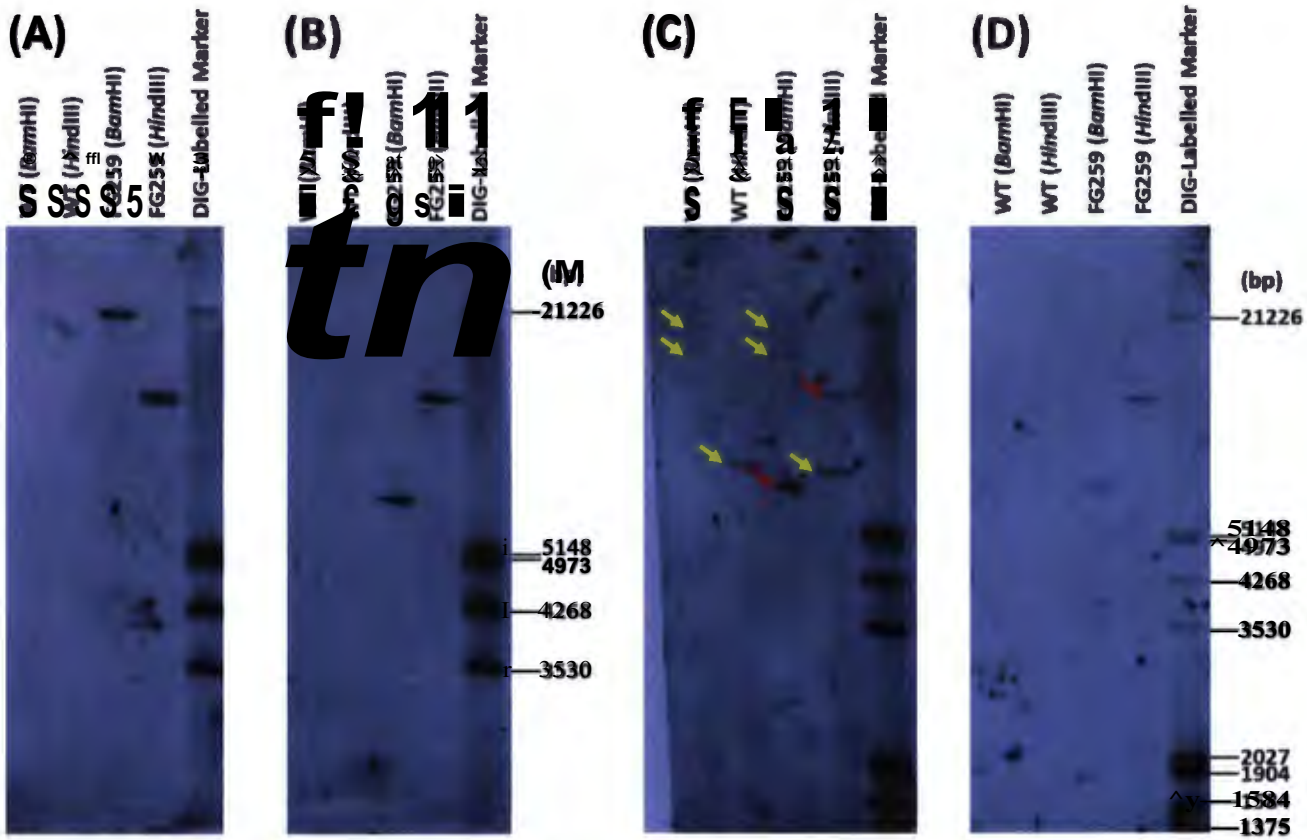
Because the ZmUbi1 promoter is a native maize sequence it is expected that additional hybridization signals corresponding to this endogenous maize genetic element would be present in addition to the bands from the T-DNA inserted in the genome of Event FG259 in blots probed with probe C that contains the ZmUbi1 promoter element. The Southern blot hybridized with probe C that includes the ZmUbi1 promoter sequence is shown in Figure 6. A hybridization fragment of ~6 kb is apparent in DNA from Event FG259 digested with *BamHI*, and a hybridization fragment of ~ 10 kb is apparent in DNA from Event FG259 digested with *HindIII*. These fragments are predicted from the location of the *BamHI* and *HindIII* restriction sites in the T-DNA and flanking maize genomic DNA [Figure 4a). As expected, additional hybridization fragments were seen in both digests when probed with probe C that contains the ZmUbi1 promoter. In the *BamHI* digest, two fragments [~13 and 16 kb) were present in both WT [control) and Event FG259

DNA, while in the *HindIII* digest a 7 kb fragment is present in both. The predicted and observed hybridization fragments from these Southern blots are listed in Table 4, and confirm that Event FG259 contains a single T-DNA insert within its genome.

Table 4. Summary of Southern blot analysis of transgenic maize line FG259. The expected and observed fragment sizes from probing *BamHI* and *HindIII* digested DNA with probes A, B, C, and D are presented. Asterisks (*) designate endogenous bands that were also found in the wild-type control that are predicted to contain the maize ZmUbi1 promoter.

Probe	Sample	Predicted Fragment Size	Observed Fragment Size
A	FG259 (*BamHI*)	Unknown (>3,849 bp)	≈20 kbp
A	FG259 (*HindIII*)	9,933bp	≈10 kbp
B	FG259 (*BamHI*)	6,146 bp	≈6kbp
B	FG259 (*HindIII*)	9,933 bp	≈10 kbp
C	FG259 (*BamHI*)	6,146 bp	≈6, 13*, 16*
C	FG259 (*HindIII*)	9,933 bp	≈10, 7*
D	FG259 (*BamHI*)	6,146 bp	≈6
D	FG259 (*HindIII*)	9,933 bp	≈10

Figure 6. Southern blot of restriction endonuclease-digested Event FG259 DNA and WT control DNA hybridized with (A) probe A covering the GTL promoter region, [B) probe B covering the AC1 region, (C) probe C covering the ZmUbi1 promoter region, and (D) probe D covering the PMI region. In panel C, T-DNA hybridization bands in FG259 digest by *Bam*HI and *Hin*dUI are indicated by red arrows. WT and FG259 DNA digest bands resulting from hybridization with the endogenous ZmUbi1 promoter are indicated by yellow arrows.



6.1.5.2 Screening for the absence of plasmid vector backbone fragments

The potential for the transfer of genetic elements derived from the plasmid vector (b) (4) that lie outside of the T-DNA borders (plasmid backbone) to the genome of Event FG259 was investigated using Southern analysis. Probes corresponding to the *ori* (ColE1 origin of plasmid replication), *sat* (streptothricin acetyltransferase), and *aadA* (aminoglycoside adenyltransferase) genetic elements (Figure 4b) were used in Southern blots containing restriction endonuclease digested genomic DNA of the AC1 Glucanase-expressing maize Event FG259. None of the DNA fragments derived from the vector portion outside of the T-DNA (backbone) of plasmid (b) (4) demonstrated hybridization to genomic DNA from the AC1 Glucanase-expressing maize Event FG259 (Figure 7). This result demonstrates the absence of DNA fragments derived from the vector backbone portion of plasmid (b) (4) in the genome of maize Event FG259.

A separate Southern hybridization study was conducted using a hybridization probe consisting of the entire (b) (4) vector backbone outside the LB and RB sequences of the T-DNA (probe E in Figure 4b). A Southern blot of restriction endonuclease digested genomic DNA of Event FG259 and plasmid (b) (4) vector DNA hybridized with probe E is presented in Figure 8. The absence of hybridization to the DNA from Event FG259 and the confirmation of the expected hybridization to the vector (b) (4) DNA confirm that the genome of Event FG259 does not contain elements derived from the vector backbone of plasmid (b) (4).

Figure 7. Southern blot of Event FG259 genomic DNA hybridized with (b) (4) vector backbone probes containing the ColE1, *aadA*, and *sat* gene elements..

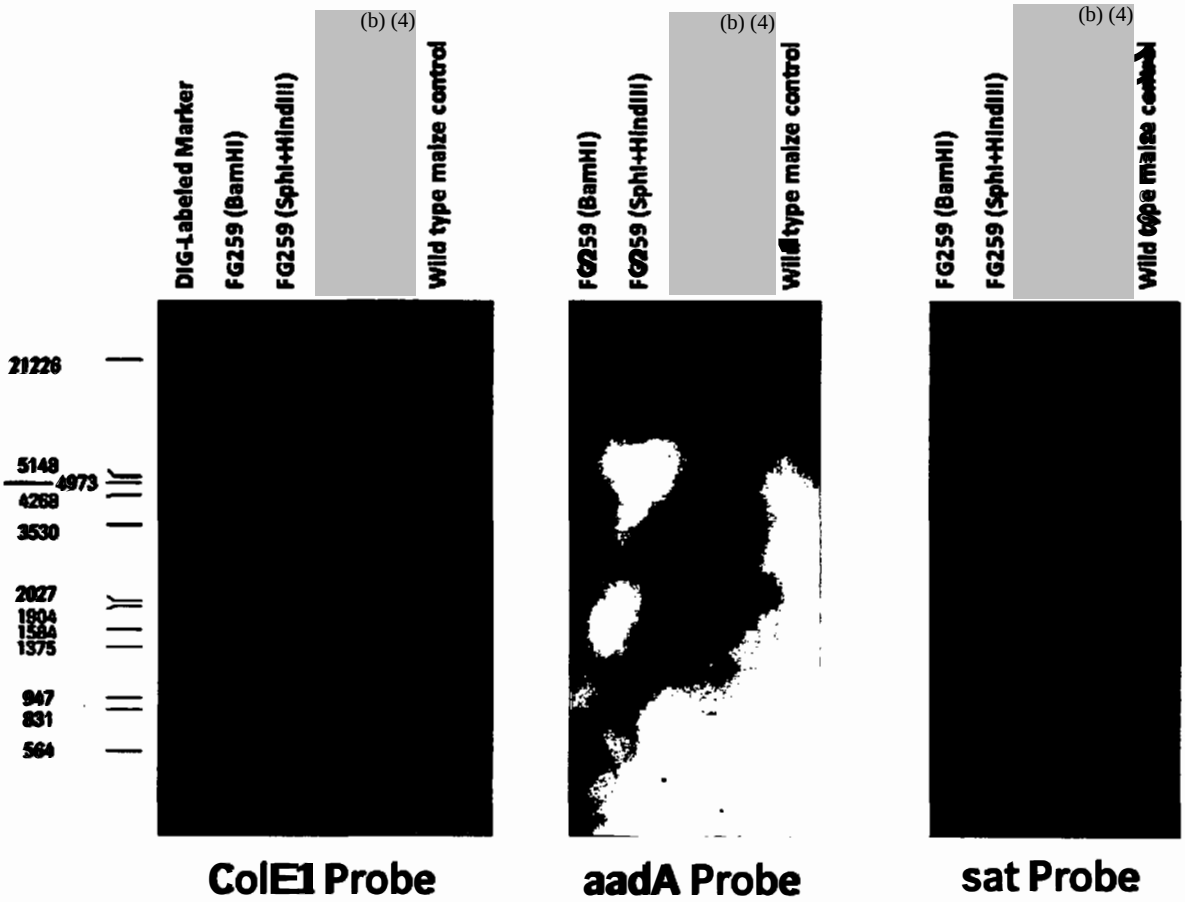
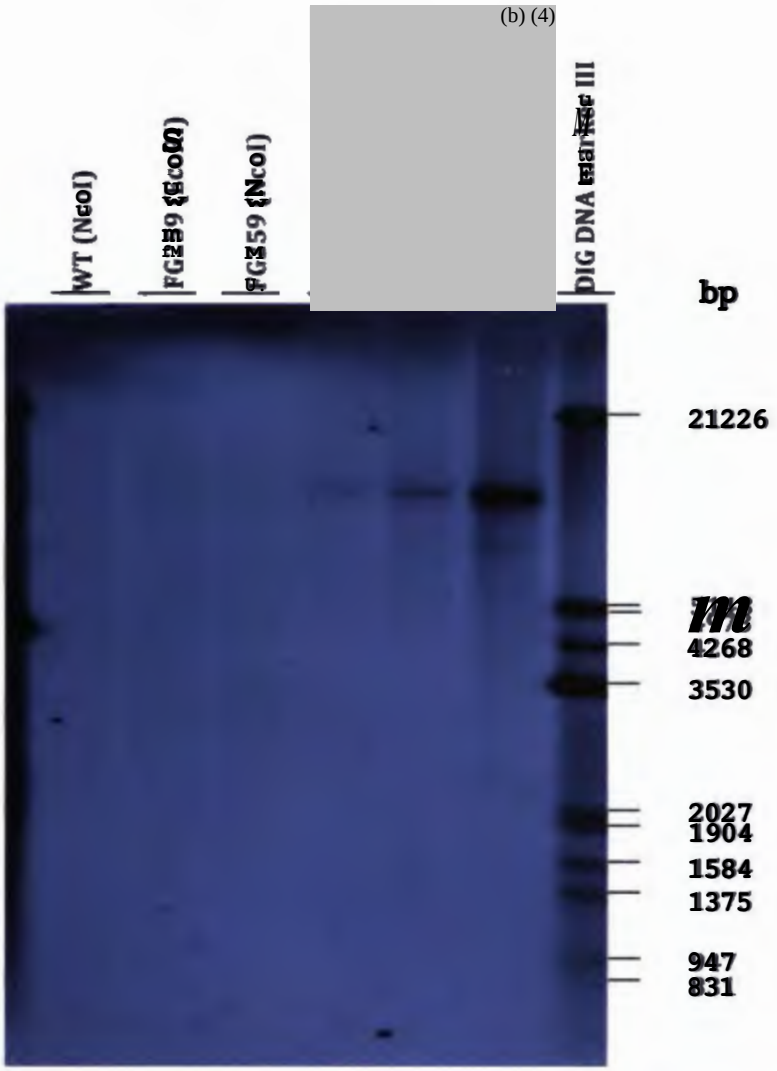


Figure 8. Southern blot of genomic DNA of Event FG259 hybridized with probe E that contains the entire T-DNA backbone region of plasmid (b) (4). The Southern blot included two lanes of Event FG259 genomic DNA, one digested with EcoRI and another digested with NcoI. One third copy, one copy and three copy equivalent amounts of the plasmid (b) (4) DNA and genomic DNA from conventional, nontransgenic, maize digested with NcoI were included as controls. As expected, the 8.9 kb NcoI DNA fragment of plasmid (b) (4) hybridized with probe E in the positive control digestions.



6.1.5.3 Characterization of the T-DNA chromosomal integration site in Event FG259

The complete nucleotide sequence of the T-DNA insertion and flanking maize genomic DNA in Event FG259 was determined. Comparison of this sequence with the sequence of the T-DNA in the transforming plasmid (b) (4) determined that, with the exception of a partial left border repeat of (b) (4) nucleotides, the coding sequences of the *AC1* Glucanase and *manA* genes in the T-DNA were not altered during the transformation process and are identical to the sequences of these genes in (b) (4).

The compete nucleotide sequence and a genetic map of the T-DNA locus of Event FG259 incuding the flanking maize genomic DNA are presented in Appendix 1 and Figure 4a, respectively. Sequence analysis of the regions at the junction of the T-DNA and maize genomic DNA was conducted in order to identify novel putative open reading frames that span the junctions between the Event FG259 T-DNA and the flanking maize genomic DNA. The nucleotide sequence of the maize genomic DNA adjacent to the RB flank of the T-DNA was sequenced to a distance 1478 bp from the insertion and the LB flank was sequenced to a distance of 1597 bp from the insertion. The sequences of the T-DNA junction regions of Event FG259 were analyzed at both ends of the T-DNA insertion in all six reading frames for any putative new open reading frames (ORF). A minimum translation size of 30 amino acids was used in this analysis as proposed by Harper *et al.* (2012). Furthermore, only new ORFs that were created between the T-DNA ends and the maize genomic DNA flanking sequences as a result of the T-DNA insertion were considered in the analysis. The identified ORFs were subsequently analyzed for amino acid sequence homology to known protein toxins.

At the right T-DNA border a novel 96 bp ORF encoding a putative peptide of 31 amino acids was created as a result of the T-DNA insertion. This (b) (4) is positioned on the minus DNA strand of the Event FG259 T-DNA and its nucleotide and deduced amino acid sequences are shown below. The (b) (4) putative peptide does not have any significant similarities to known proteins in the NCBI database when it is used as a query in the BLASTP search under default program settings. No novel ORFs were identified at the left border junction of the T-DNA with the maize genomic DNA flanking sequence in Event FG259.

(b) (4)

The maize genomic DNA flanking the T-DNA insertion in Event FG259 was analyzed in order to identify the genomic location of the insertion and endogenous maize genes in the region of the insertion. The sequence of the maize genomic DNA at the RB, including 1478 bp of sequence extending from the site of the T-DNA insertion was used as a query at the default settings in a BLASTN search of the publicly available maize B-73 reference genome database (assembly B73 RefGen_v4). Multiple areas of high identity to this sequence from all 10 maize chromosomes were identified, including 18 regions with 98% identity or greater. This result indicates that the insertion is located in an area of the genome that contains a highly repetitive sequence. Similarly, 1597 bp of nucleotide sequence derived from the LB junction of the T-DNA in Event FG259 was queried against the B73 maize genomic seqeunce. The results of this query were similar to that of the RB flanking sequence returning 307 sequences with 95% or greater identity with multiple maize chromosomes. These results confirm the finding from the query with the RB flanking sequence that the T-DNA of Event FG259 is inserted in a region of the maize genome that contains a highly repetitive sequence. The complete nucleotide sequence of the T-DNA locus of Event FG259 is presented in Appendix 1.

In order to identify the T-DNA chromosomal insertion site of Event FG259, a study using marker analysis was conducted. Marker analysis examines backcross progeny to determine the chromosome and chromosomal segment biased for retained markers associated with the segregating genetic background in which the original transformation was conducted (maize line (b) (4)). In this analysis, backcrossed progeny of Event FG259 were selected for the presence of the T-DNA locus containing the *AC1* Glucanase gene and these were analyzed for the presence of known genetic markers from the maize genome. Those markers that are closely linked to the chromosomal T-DNA integration site will be retained at a greater frequency due to their proximity to the T-DNA integration site. To determine the location and frequency of retained markers in selected transgenic progeny, nine independent transgenic AC1 progeny that resulted from two rounds of backcrossing (BC2) Event FG259 with maize inbred G as the recurrent parent were analyzed. A total of 3017 single nucleotide polymorphisms (SNP) (1136 of which were polymorphic between (b) (4) and inbred G) were analyzed following use of the Illumina 55k chip and for which the maize Version 4 genome map annotation (Illumina, 2018] was used.

Analysis of the backcrossed progeny indicated that the T-DNA insertion is located in a 6.6Mb segment of chromosome (b) (4) that includes SNP markers (b) (4) and (b) (4) (corresponding to chromosome (b) (4) positions (b) (4) and (b) (4), respectively]. The maize genomic database shows that there is a region within this segment that is homologous to the contiguous sequence identified to be the insertion site according to the flanking sequence determined for the AC1 Event FG259. There were no other polymorphic markers that showed this 100% bias for the (b) (4) genetic markers on any other chromosome or chromosomal segment. These observations confirm the location of the T-DNA insertion on chromosome (b) (4) in event FG259.

6.1.5.4 Genetic stability of the insert over multiple generations

The stability of the T-DNA insertion in Event FG259 was determined using Southern blots over multiple generations of progeny of maize Event FG259. Genomic DNA from four backcross (BC) generations of an F1 hybrid of Event FG259 and maize inbred E was digested with BamHI and run on an agarose gel prior to blotting to nylon membrane. Also included in the gel was 1 and ¼ genome equivalents of *EcoRI* restricted (b) (4) DNA and the *manA* gene probe fragment DNA as positive controls. The backcross generations that were examined in this study are designated BC1E, BC2E, BC3E, and BC4E (see the breeding diagram in Figure 1). Hybridization of a T-DNA PMI *manA* gene probe (probe D in Figure 4b) to *BamHI* digested genomic DNA from the four Event FG259 BC generations demonstrated the hybridization of an approximately 6000 bp fragment that contains the *manA* gene from the T-DNA insertion, the LB, and the LB maize flanking DNA (Figure 9), thereby confirming the generational stability of the Event FG259 T-DNA insertion. As expected, the DNA probe in this study failed to hybridize to fragments in the nontransformed conventional maize DNA control, whereas the (b) (4) and probe DNAs demonstrated hybridization to the expected size fragments (Figure 9 and Table 5).

Figure 9. Southern blot of genomic DNA from four BC generations of Event FG259 (BC1E, BC2E, BC3E, BC4E), conventional maize genomic DNA, 1 and ¼ genome equivalents of EcoRI digested (b) (4) DNA and probe DNA hybridized with a T-DNA PMI *manA* gene probe.

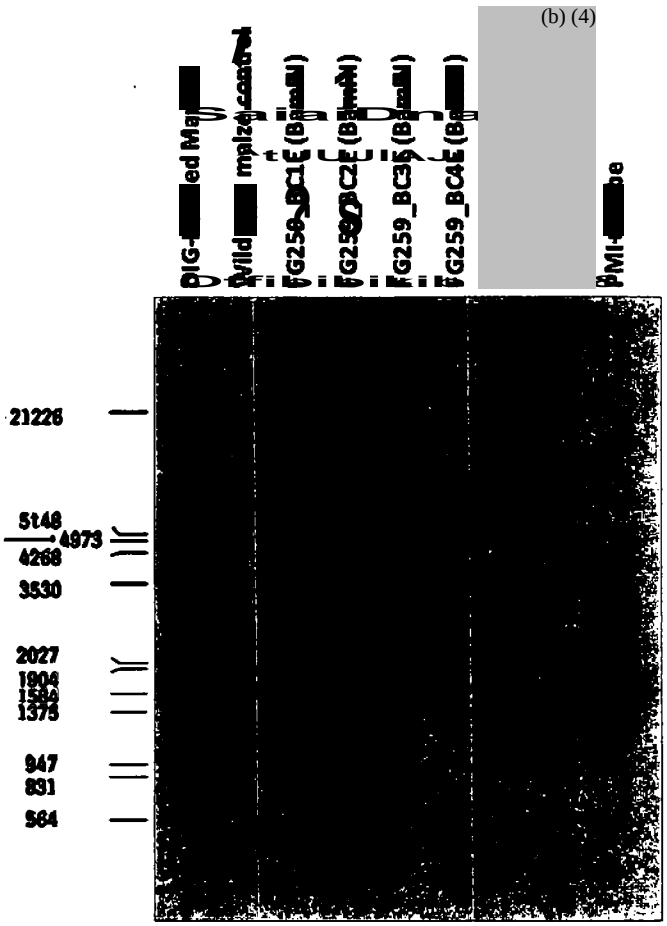


Table 5. Predicted and observed DNA fragment sizes for a Southern blot of BamHI digested genomic DNA from four BC generations of Event FG259 with the T-DNA *manA* gene probe.

Sample	Predicted Fragment Size	Observed Fragment Size
FG259 (*BamHI* digested)	6,195 bp	~6,000 bp
(b) (4) (*EcoRI* digested) 1 & ¼ copy	8,748 bp	~9,000 bp
Wild type maize control	--	--

6.1.5.5 Mendelian inheritance of the AC1 gene in Event FG259

The Mendelian inheritance of the AC1 Glucanase gene in maize Event FG259 was confirmed using segregation analysis of genotyped plants. Event FG259 maize plants of the F1 generation that were hemizygous for the *AC1* Glucanase gene were backcrossed (BC) four times to maize inbred line E (BC1E, BC2E, BC3E, and BC4E; see breeding diagram (Figure 1, §6.1.3) that lacks the T-DNA insertion. Seed from each backcross generation were germinated and DNA was isolated from the plant tissues. DNA was genotyped using locus-specific genotyping primers and segregation of the AC1 Glucanase gene was calculated. The results presented in Table 6 demonstrate that inheritance of the AC1 Glucanase gene did not deviate significantly from the expected segregation of a single locus (50%), confirming the inheritance of the T-DNA in Event FG259 over four generations in the expected Mendelian pattern. These results provide further confirmation that there is a single T-DNA that contains the AC1 gene in the genome of Event FG259.

Table 6. Segregation of the FG259 genetic locus in four backcross generations.

Generation	Plants (#)	FG259 Positive Plants	FG259 Negative Plants	FG259 Positive (%)	CHI Square test (p-value)
BC1E	48	28	20	58.3	0.248
BC2E	54	27	27	50.0	1.000
BC3E	48	21	27	43.8	0.387
BC4E	68	28	40	41.2	0.146

6.2 Characterization and safety of the AC1 Glucanase

6.2.1 Introduction to β-glucanase enzymes

β-glucanase enzymes are ubiquitous in nature. In most plants they play a role in the opening of plasmodesmata channels that connect adjacent plant cells (Levy *et al.*, 2007; Leubner-Metzger, 2003) and they are produced in the germinating seeds of plants (Vögeli-Lange *et al.*, 1994), including tomato (Chun-Ta *et al.*, 2001), barley (Leah *et al.*, 1995), peanut (Liang *et al.* 2005), wheat (Moravčíková et al., 2016) and many others. In addition, β-1,3-glucanases attack critical components of fungal cell walls and are expressed in many plants as part of a broad generalized defense mechanism against fungal pathogenesis (Boller, 1987; Collinge and Slusarenko, 1987; Cornelissen and Melchers, 1993). Therefore, it can be concluded that glucanase enzymes are present in many different plant-derived food ingredients. β-glucanases from various sources are also widely used as food processing aids in the production of fermented beverages such as beer and wine, and in the production of yeast extract.

Glucanase enzymes have been used for many years in animal feed to increase the digestibility of the feed for monogastric animals (Bedford and Classen, 1992; Burnett, 1966). For the same purpose, glucanase enzymes are components of digestive aids marketed to humans and they appear in many such products, including Veganzyme® [Global Healing Center, 2017), Digestive Enzymes Ultra [Pureformulas, 2017), and βeta-1,3D Glucan (LifeExtension, 2017). Glucanases and other carbohydrase enzymes have been used for many years in the production of food as processing aids for clarifying fruit and vegetable juices and beer and other alcoholic beverages (FDA, 2018; Table 7). As such, it can be concluded that there is a long history of safe consumption of glucanase enzymes in monogastric animals, including poultry, swine and humans.

Table 7. A partial list of β-glucanases and related carbohydrases that have been approved by FDA as GRAS for use in food production in the past 5 years (FDA, 2018).

GRAS No.	Enzyme	Source Organism	GRAS Use
592	β-glucanase	*Bacillus subtilis*	Beer processing
584	Cellulase	*Penicillium funiculosum*	Processing for beer and baking
566	Mannanase	*Trichoderma reesei*	Oil, fruit, vegetable processing; coffee production
535	β-glucanase	*Streptomyces violaceoruber*	Processing of yeast extract and alcoholic beverages
482	β-glucanase and Xylanase	*Disporotrichum dimorphosporum*	Production of beer and fermented beverages
479	β-glucanase, cellulase and Xylanase	*Talaromyces emersonii*	Production of beer and fermented beverages

6.2.2 AC1 Glucanase product characterization

The AC1 Glucanase is a 37.7 kDa endo-1,4-β-glucanase that possesses 96% identity to the Glycosyl Hydrolase Family 5 Cel5A cellulase from *Thermotoga maritima*, originally isolated from geothermally heated marine sediments (Huber *et al.*, 1999). The gene encoding the AC1 Glucanase was isolated from an environmental DNA library, where it was found to encode a protein that was identical to Cel5A from *T. maritima*. This sequence was further modified by GSSM for improved thermostability to generate the AC1 Glucanase gene.

For the purpose of characterizing the AC1 Glucanase product, protein extracts prepared from grain derived from three representative AC1 Glucanase product

batches (Lot numbers AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077) were assessed. The product batch numbers, location of planting and dates of planting and harvest are shown in Table 8, and further details of the characterization of the AC1 Glucanase product are described in Appendix 2. Planting of AC1 Glucanase-expressing maize Event FG259 and harvest of the grain were performed using commonly used agronomic practices for maize. Cultivation of maize Event FG259 utilized common agronomic practices for maize including the use of fertilizers, herbicides and pesticides approved for use on maize. (b) (4)

AC1 protein extracts were prepared as follows. Twenty grams of AC1 maize flour was suspended in 100 ml of 60°C extraction buffer (100 mM sodium phosphate, 0.01% Tween 20, pH 6.5) and shaken at 60°C for 1 hour. Duplicate samples from each product batch were prepared. Aliquots of about 1.2 ml of the sample suspension were transferred into 1.5 ml tubes and centrifuged at 16,000 g for 10 minutes. The supernatant was reserved for analyses.

Table 8. Planting locations and dates for the production of three representative Glucanase AC1 product batches.

Product Batch No.	AV_AC1_0070	AV_AC1_0075	AV_AC1_0077
Planting Location	(b) (6)	(b) (6)	(b) (6)
Planting Date	11/28/2016	1/20/2016	11/28/2016
Harvest Date	4/27/2017	5/16/2016	4/27/2017

6.2.3 Specific activity of AC1 Glucanase

AC1 Glucanase activity was assayed according to an Agrivida, Inc. SOP described in Appendix 3. Since β-glucanase activity is the primary activity of the AC1 Glucanase, a colorimetric β-glucanase assay was used to determine AC1 enzyme activity. The glucanase colorimetric assay uses a commercial substrate consisting of an azurine-cross linked barley β-glucan (AZCL-Beta-Glucan; Megazyme, 2012). Hydrolysis of this substrate by the AC1 Glucanase produces water-soluble dye fragments that can be measured by absorbance at 590 nm (A590), providing a measurement that correlates directly with enzyme activity. The protein extracts produced from the Event FG259 grain flour samples as described above were diluted 60- and 80-fold and 50 µl of each dilution was mixed with 450 µl of AC1 Glucanase extraction buffer containing the AZCL-Beta-Glucan substrate. The mixtures were incubated in a water bath at 80°C for 1 hour and mixed with 1 ml of 2% (w/v) Tris base to

terminate the reaction. The reaction was centrifuged at 3,000 x g for 10 minutes, and 100 µl of supernatant was removed to a microplate to measure the increase in absorbance at 590nm (A590). AC1 Glucanase activity (A590/mg) was determined for each batch by dividing the A590 by the amount of flour (mg) included in the protein extract. The enzyme activity measured within one hour (A590/mg flour) can also be converted to glucanase activity units (µmol of reducing sugar equivalents released/min/g flour) by multiplying by 9 (Appendix 3).

Quantification of AC1 Glucanase protein in flour extracts was determined immunologically using an ELISA method following the protocol from (b) (4). (b) (4) (b) (4) (b) (4) .. A series of dilutions of AC1 Glucanase protein that had been purified from grain derived from AC1 Glucanase-expressing maize Event FG259 was used to construct a standard curve for calculation of AC1 Glucanase protein content in the aqueous extracts from each product batch. For quantification of AC1 Glucanase protein, flour extracts prepared as described above were diluted 60,000- and 80,000-fold. The specific activity of AC1 Glucanase in the test substance material from each product batch was determined and is expressed in two forms (units β-1,4-glucanase activity/mg AC1 protein and A590/mg AC1 protein). The specific activity of the AC1 Glucanase from the three product batches is presented in Table 9.

Table 9. Specific activity determined for the AC1 Glucanase in three representative product batches.

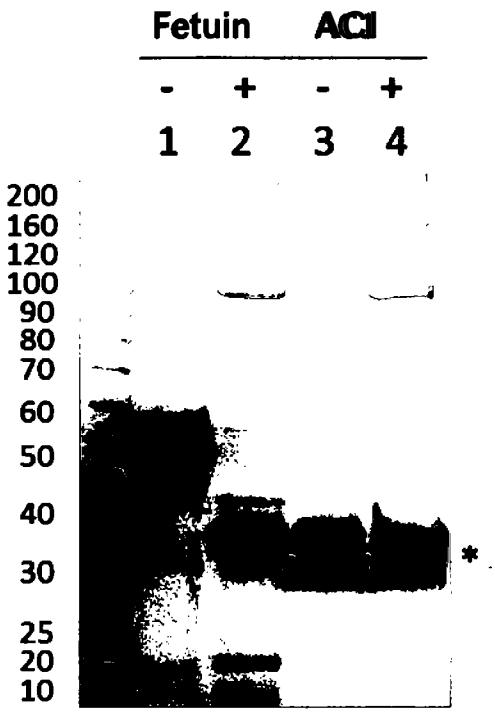
	A590/mg of AC1 protein		Units/mg of AC1 protein	
Product Batch	Average	Stdev	Average	Stdev
AV_AC1_0070	32512.49	42.60	292.61	0.38
AV_AC1_0075	42685.91	2843.86	384.17	25.59
AV_AC1_0077	33486.58	894.77	301.38	8.05

6.2.4 Assessment of the glycosylation status of the AC1 Glucanase

The glycosylation status of the AC1 Glucanase protein produced in maize Event FG259 was examined using a protein deglycosylation kit obtained from (b) (4) (b) (4) and following the protocol supplied with the kit. Briefly, the AC1 Glucanase protein purified from a protein extract prepared from a typical GraINzyme® AC1 Glucanase product batch along with a positive control consisting of fetuin, a bovine glycoprotein possessing both N- and O-linked glycosyl moieties, was treated with the deglycosylating enzymes PNGase F and O-glycosidase, which act to remove N- and O-linked glycosyl groups, respectively. After treatment with these deglycosylating enzymes, treated and untreated protein extracts were examined by SDS-PAGE and the apparent size of the protein in each was compared. In the case of

glycosylated proteins, removal of the glycosyl moieties results in an apparent reduction in the size of the protein on SDS-PAGE gels. SDS-PAGE gels containing enzyme-treated and untreated total protein extracts from grain of maize Event FG259 are shown in Figure 10. The results show that there is no change in the apparent size of the AC1 protein in response to enzyme treatment, demonstrating that AC1 protein produced in the grain of maize is not glycosylated.

Figure 10. Evaluation of AC1 Glucanase glycosylation status. The positive control, bovine fetuin (lanes 1 and 2), is a glycoprotein bearing both N- and O-linked glycosyl moieties. Fetuin and AC1 (lanes 3 and 4) were treated with deglycosylating enzymes (+, lanes 2 and 4), and their molecular weights compared to that of untreated samples. The reduction in the apparent molecular weight of fetuin after treatment demonstrates that the deglycosylation reaction was functional. No difference in size was detected in response to treatment of AC1 with deglycosylating enzymes, indicating that AC1 is not glycosylated when produced in maize. Proteins were separated on a 4-12% SDS-PAGE gradient gel.



6.2.5 Confirmation of the AC1 Glucanase amino acid sequence

As noted in §6.1.3 and Figure 2, the AC1 gene expression construct was designed such that the AC1 Glucanase protein includes the (b) (4) amino acid *Zea mays* (b) (4) (b) (4). The (b) (4) directs the AC1 Glucanase protein to the endoplasmic reticulum (b) (4). The (b) (4) has been successfully used to target

numerous heterologous proteins to the endoplastic reticulum (de Virgilio *et al*, 2008; Harrison *et al*, 2011; Torrent *et al*, 2009]. The signal sequence is removed from the expressed precursor protein by a signal peptidase during co-translational transport into the endoplasmic reticulum, generating the mature protein. The signal peptidase cleaves the signal peptide at a sequence proximal to its C-terminal junction with the N-terminus of the mature amino acid sequence. However, the fidelity with which the signal peptidase recognizes the target sequence and the precision with which it cleaves the polyeptide at the predicted site may vary, with actual cleavage products reported to vary up to two amino acids N- or C-terminal to the predicted cleavage site (Park *et al*, 2017].

AC1 Glucanase protein was purified from extracts of a typical GralNzyme® AC1 Glucanase product batch. The N-terminal amino acid sequence of the protein was determined by Edman degradation by (b) (4), (b) (6) The predicted cleavage site from literature reports of other endoplasmic reticulum proteins in maize is at Ser19, the C-terminus of the 19-amino acid signal sequence (Esen *et al*, 1982]. According to this, the signal peptide at the N-terminus of the AC1 Glucanase protein should be cleaved at the (b) (4) (Figure 11]. The N-terminal amino acid sequence of the AC1 Glucanase protein determined as described begins with the sequence of (b) (4), confirming this site as the principal product of processing. In addition, smaller amounts of mature AC1 with different N-termini were also detected that appear to result from imprecise processing of the signal sequence. In one case, processing occurs (b) (4) amino acid C-terminal to the expected site resulting in an N-terminal sequence of (b) (4) and in the other, (b) (4) amino acids N-terminal to the expected site resulting in an N-terminal sequence of (b) (4). These results confirm that the mature AC1 protein that is produced in the grain of maize has the N-terminal amino acid sequence that is expected from the coding sequence of the AC1 Glucanase gene with the exception of (b) (4). Additional confirmation of the amino acid sequence of the grain-expressed AC1 Glucanase was provided by tryptic digestion and LC/MS peptide mapping (also performed by Lake Pharma].

Figure 11. N-terminal amino acid sequence of the mature AC1 Glucanase protein produced in maize Event FG259.

(b) (4)

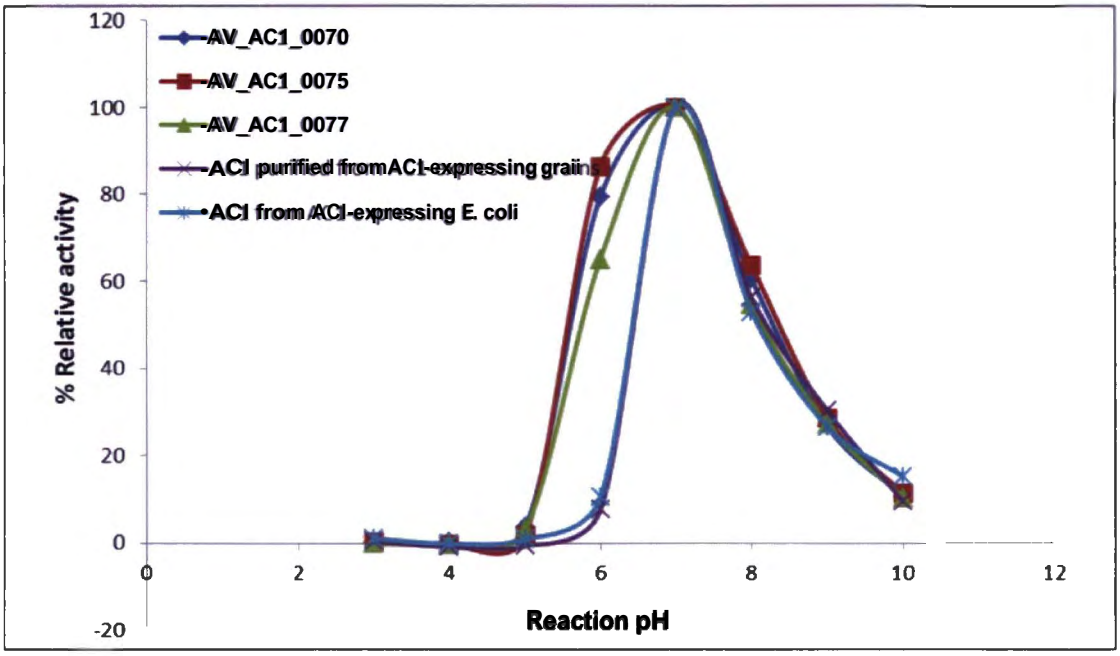
6.2.6 pH optimum of the AC1 Glucanase

The AC1 Glucanase activity in aqueous extracts from three independent AC1 product batches (Lot numbers AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077) from grain of maize Event FG259, and from *E. coli* expressing the AC1 Glucanase gene, was determined over a range of pH values to determine the enzyme's pH optimum. The AC1 Glucanase enzymatic reactions were performed in 10x CCH (0.22M citric acid, 0.44M CHES, 0.28M HEPES, pH 3) buffer that was diluted to 1x with either 1N HCl or 1N NaOH to adjust the pH from 2 to 10. Extracts of flour from the AC1 Glucanase product batches were diluted 60-fold in each 1x CCH buffer. Fifty microliters of diluted extract (the diluted purified AC1 Glucanase from grain or *E. coli*) was mixed with 1 tablet of substrate β-glucazyme ((b) (4)) and 450 l each of the 1x CCH buffers with different pH. To ensure that the reaction pH did not change upon mixing the enzyme solutions with the substrate, the reaction pH was monitored with colorpHast pH indicator strips ((b) (4)). AC1 enzyme reactions were carried out at 80°C for 1 hour before the addition of 2% Tris base to terminate the reaction.

The activity of AC1 Glucanase in protein extracts from three AC1 Glucanase product batches, and that of AC1 purified from *E. coli*, performed similarly with each preparation exhibiting maximal activity at pH 7.0 (Figure 12). Within the 3 batches of AC1 Glucanase prepared from grain, the response to pH was nearly identical, with greater than 60% activity retained at pH 6.0 to 8.0 and a rapid loss of activity at pH values greater than 8.0. Nearly identical activity profiles were generated for all preparations of AC1 Glucanase over the range of pH 3.0-10.0.

The activity profile of AC1 Glucanase purified from AC1 Glucanase-expressing *E. coli* and from the grain of one of three representative AC1 Glucanase product batches over a range of pH are similar except that the activity of the purified AC1 Glucanase is more sensitive to lower pH than the AC1 Glucanase enzyme in the crude extract from grain of the three representative product batches. Purified AC1 Glucanase exhibited only 10% activity at pH 6 compared to greater than 80% activity of AC1 Glucanase extracted from the grain (Figure 12).

Figure 12. pH optimum of the AC1 Glucanase. The glucanase activity from crude protein extracts from three representative AC1 Glucanase product batches (AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077] and of AC1 Glucanase purified from AC1-glucanase expressing grain and *E. coli* over a range of pH. Relative activity for each sample is normalized to maximal activity at pH 7.0.

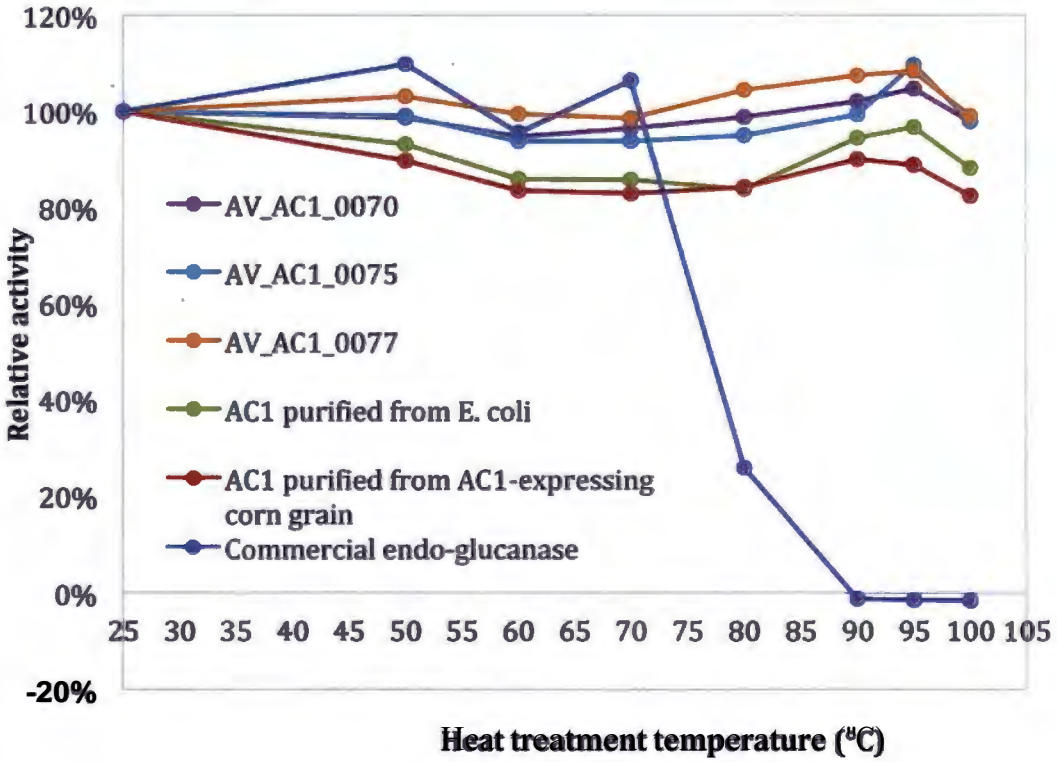


6.2.7 Thermal tolerance of the AC1 Glucanase

The thermal tolerance of the AC1 Glucanase was tested over a range of temperatures. AC1 Glucanase activity was tested in flour extracts from three representative AC1 Glucanase product batches (Lot numbers AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077), AC1-Glucanase purified from AC1-expressing *E. coli* and grain, and a commercially available thermostable β-glucanase ((b) (4) (b) (4)). AC1 Glucanase samples and the commercial β-glucanase were diluted using AC1 extraction buffer (100 mM sodium phosphate, 0.01% Tween 20, pH 6.5). Four-hundred microliters of diluted protein was placed in a shaking incubator at temperatures of 25, 50, 60, 70, 80, 90, 95 and 100°C. Incubation at each temperature was carried out for 5 min with shaking at 1000 rpm. The temperature of sample wells was checked using a Dual Channel Digital Thermometer (b) (4)). The relative glucanase activity in each of the AC1 Glucanase product batches and AC1 purified from AC1-expressing *E. coli* and grain at the different temperatures was determined and are presented in Figure 12. The AC1 Glucanase in aqueous extracts from three representative product batches demonstrated 100% activity after 5 minute incubation at temperatures from 50 - 100°C relative to its activity at 25°C. The AC1 Glucanase purified from grain or *E. coli* maintained more

than 82% activity between 60°C to 100°C. The commercial thermostable β-glucanase maintained 100% activity at 70°C relative to its activity measured at 25°C, but retained only 25.9% activity at 80°C, and then lost activity completely at temperatures of 90°C and above (Figure 13).

Figure 13. Relative glucanase activities of three representative AC1 Glucanase product batches (AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077), AC1 Glucanase purified from AC1-expressing *E. coli* and grain, and a commercial thermostable β-glucanase after 5 minutes of incubation at different temperatures.



6.2.8 Enzymatic activities of the AC1 Glucanase

AC1 Glucanase protein purified from extracts of the representative AC1 Glucanase product batch AV_AC1_0075 was tested for the presence of other significant enzymatic activities. The enzymatic activities that were tested included β-1,4-glucanase, β-1,3-glucanase, α-amylase, arabinofuranosidase, β-glucosidase, β-xylanosidase, exocellulase, endocellulase, endomannanase, endoxylanase, and pectinase. The detectible enzymatic activities of the AC1 Glucanase and a positive control enzyme with the corresponding activity (commercial enzyme) were compared for each enzymatic assay tested. The results show that the primary activity of AC1 protein is β-1,4-glucanase. AC1 Glucanase also possesses significant

endocellulase and exocellulase activities in addition to detectable levels of β-1,3-glucanase, β-xylanosidase and endomannanase activities (Table 10).

Table 10. Enzymatic activities of protein extracts of grain derived AC1 Glucanase. The activities shown are the average of three measurements and are expressed in arbitrary units with the standard deviation (SD) of the measurements. Control reactions incorporating commercially-available enzymes with the specified activity were also run to demonstrate the functionality of each assay. Enzyme assays were conducted at 37°C and 80°C at pH 6.5.

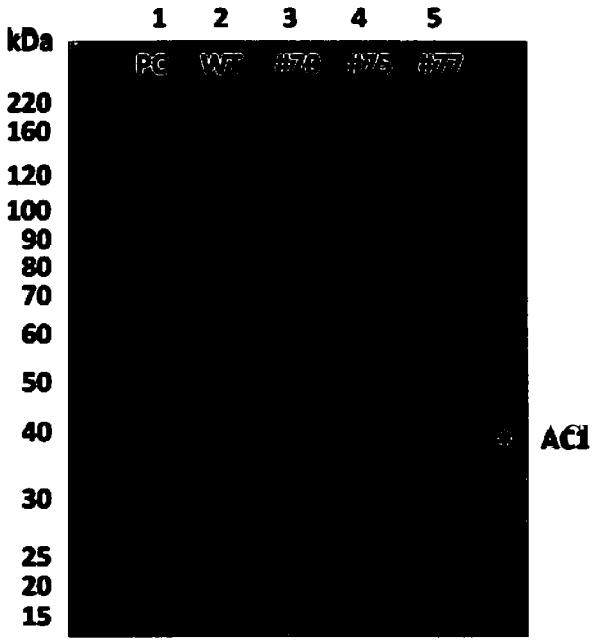
Enzyme activity (U/mg protein)	AC1, 37°C		PC*, 37°C		AC1, 80°C		PC, 80°C	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
β-1,4-glucanase	12.120	12.060	0.140	0.004	281.520	10.925	1.189	0.056
Endo-cellulase	10.080	0.990	0.222	0.007	178.200	2.718	0.283	0.024
Cellobiohydro-lase (Exo-cellulase)	0.192	0.004	0.001	0.000	0.907	0.015	0.000	0.000
Endo-mannanase	0.468	0.190	0.762	0.015	17.406	0.108	25.341	4.237
Endo-β-1,3-glucanase	0.005	0.000	83.873	5.799	0.001	0.000	21.272	4.879
β-xylosidase	0.001	0.000	0.223	0.022	0.001	0.000	0.038	0.005

*PC, Positive controls; β-1,4-glucanase (Sigma G8673); Endo-cellulase (Sigma 22178); Cellobiohydrolase [Exo-cellulase, CBH I] (Megazyme E-CBH); Endo-mannanase (Megazyme E-BMATM); Endo-β-1,3-glucanase (Megazyme E-LAMSE); β-xylosidase (Sigma X-3501).

6.2.9 Molecular weight and Immunoreactivity of AC1 Glucanase

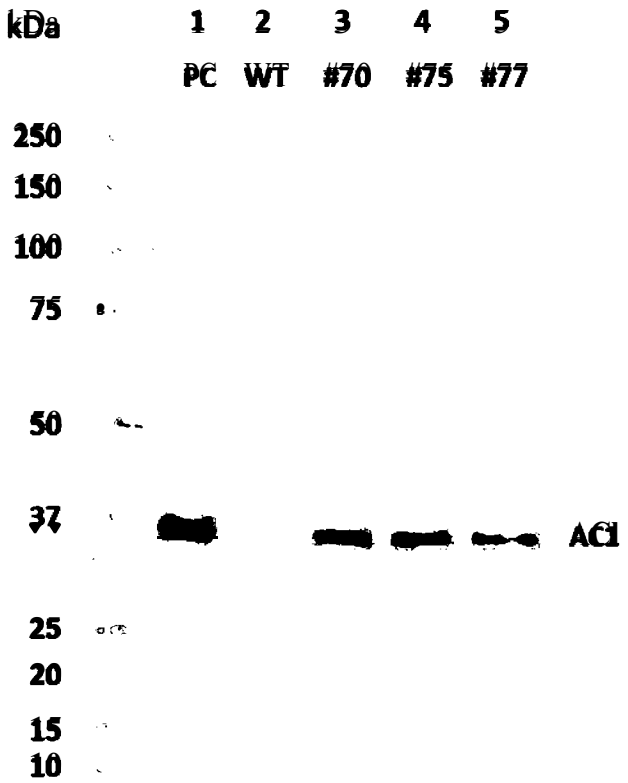
An SDS-PAGE gel containing protein extracts from grain flour of each of the three representative AC1 Glucanase product batches (AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077) and from a conventional maize variety not engineered to produce the AC1 Glucanase were stained with Coomassie blue to enable visualization of the proteins. Examination of the gel and comparison of the samples demonstrated that there is a prominent protein band in the extracts from all three representative product batches that is absent in the extract from the conventional maize flour and that has the same molecular weight as the AC1 Glucanase protein that was produced and purified from the grain of AC1 Glucanase-producing maize (Figure 14). Comparison of the position of these protein bands in the gel relative to the protein molecular weight markers included on the gel shows that the prominent protein band in the extracts of the representative product batches and the purified AC1 Glucanase protein are approximately 37 kilodaltons (kDa) in size. This estimation of the size of the protein bands compares well with the predicted size of 37.7 kDa (http://web.expasy.org/compute_pi) for the mature AC1 Glucanase protein that includes the endoplasmic reticulum retention signal from maize (SEKDEL).

Figure 14. Coomassie-blue stained SDS-PAGE gel containing protein extracts from three AC1 Glucanase product batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77), extract from grain of a conventional nontransgenic maize variety (WT, lane 2), and purified AC1 Glucanase protein from AC1 Glucanase-expressing maize grain (PC, lane 1). Protein size markers ((b) (4) (b) (4)) were run in the left lane and their sizes in kDa are indicated on the left side of the gel. An asterisk indicates the position of AC1 Glucanase protein present in aqueous extract from each of the AC1 Glucanase product batches.



In order to assess the immunoreactivity and integrity of the AC1 Glucanase in Event FG259, Western blot analysis of the proteins extracted from grains of the three representative AC1 Glucanase product batches, from grains of a conventional non-transgenic corn (WT), and AC1 Glucanase protein purified from AC1 producing grain was performed using mouse monoclonal antibody generated against the AC1 Glucanase protein manufactured by (b) (4). The results revealed the presence of one immunoreactive protein corresponding to the predicted molecular weight of the AC1 Glucanase protein [*ca.* 37.7 kDa; Figure 15) in all samples derived from Event FG259. These results confirm the integrity, identity, and molecular weight of the prominent AC1 Glucanase protein that is present in each of the three representative product batches. This protein is absent in conventional maize.

Figure 15. Western blot of protein extracts that was reacted with an AC1-specific antibody. The samples on the Western blot include protein extracts of three representative AC1 production batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77), the extract from grain of a conventional AC1 non-expressing maize variety (WT; lane 2) and purified AC1 protein from AC1 expressing corn grain (PC, lane 1). Protein size markers (b) (4) were run in the left lane and their sizes in kDa are indicated on the left side of the gel.



6.2.10 Assessment of Amino Acid Homology of AC1 Glucanase to Known Protein Toxins

A global sequence similarity search of the AC1 Glucanase amino acid sequence was conducted on January, 16, 2019 against the NCBI Protein dataset using the BLASTP algorithm (Altschul et al., 2005). A sequence file comprising the translation of the *AC1* Glucanase gene was queried using the BLASTP 2.6.1 algorithm against the "nr" dataset, which incorporates non-redundant entries from all GenBank nucleotide translations along with protein sequences from SWISS-PROT, PIR, PRF, and PDB.

A cutoff expectation (E) score of 1.0 was used to generate biologically meaningful similarity between the AC1 Glucanase protein and proteins in the "nr" dataset. Although a statistically significant sequence similarity generally requires a match with an E score of less than 0.01 (Pearson, 2000), a cutoff of E < 1.0 ensures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 5000.

The top 5000 proteins with homology to the AC1 Glucanase protein with an E score of less than 1.0 were examined. A large number of the accessions returned by the search displayed complete significance (E = 0) and represented nearly identical or closely related glucanase proteins from various microbial species. Most of the remaining sequences represented a variety of proteins that were all functionally related bacterial glycoside hydrolase enzymes such as endoglucanases and cellulases. None of the proteins were known toxins or proteins with toxicity related activities. This demonstrates that the AC1 Glucanase protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be toxic.

6.2.11 Evaluation of the Safety of the AC1 Glucanase

The AC1 Glucanase is a well-characterized glucanase enzyme with well-known enzymatic activities. Very few enzymes in nature are orally toxic and those that are toxic are so due to their specific biological mode of action. Glucanase and related cellobiohydrolase enzymes are known not to be orally toxic. This is supported by the fact that humans have experienced oral exposure to glucanase enzymes as they are naturally present in most plants, including maize, wheat, barley, peanuts, tomatoes and other food plants. Glucanase enzymes are also expressed in microbes such as yeasts and bacteria that are used in the production of common foods, and they are produced by many of the organisms comprising the human gastrointestinal microbiome. In addition, glucanases are marketed as human dietary supplements to improve digestion and there have been no reports or incidents of toxicity as a result of this exposure. As a result, it can be concluded that humans and animals have had a long history of dietary exposure to glucanase enzymes without any indication of toxicity. The biological activity of the AC1 Glucanase is restricted to degradation of NSP substrates, and the only other dietary impact of its presence in food is the same as any other dietary protein that is digested to its constituent amino acids. A search of the nonredundant protein databases with the AC1 Glucanase amino acid sequence revealed that the AC1 Glucanase protein has no similarity to any known toxic proteins.

With consideration of the above points, the decision tree for establishing the safety of food processing enzymes as described by Pariza and Johnson (2001)

was applied to the AC1 Glucanase. The results of this analysis are presented in Table 11. Since glucanases have a long history of safe use in animal feed and in human dietary supplements and since the maize production host is known to be safe, the decision tree determined that the AC1 Glucanase is safe and that animal toxicity studies are not required to demonstrate its safety as a food or feed enzyme. In conclusion, there is sufficient information about the AC1 Glucanase to conclude that it is safe for its intended use as a feed additive for poultry.

Table 11. Application of the decision tree for determining the safety of food processing enzymes that was developed by Pariza and Johnson (2001) to the AC1 Glucanase.

	Question?	AC1
1	Is the production strain genetically modified? If yes go to 2.	YES
2	Is the production strain modified using rDNA? If yes go to 3a.	YES
3a	Do the enzyme products have a history of safe use in food or feed? If yes go to 3c.	YES
3c	Is the test article free of transferable antibiotic resistance gene DNA? If yes go to 3e.	YES
3e	Is all other introduced DNA well characterized and free of attributes that would render it unsafe? If yes go to 4.	YES
4	Is the introduced DNA randomly integrated into the genome? If yes go to 5.	YES
5	Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins will not arise due to the genetic modification? If yes go to 6.	YES
6	Is the production strain derived from a safe lineage? If yes the test article is ACCEPTED	YES

6.2.12 Conclusions on the Safety of AC1 Glucanase

Bioinformatic comparison of the amino acid sequence of AC1 Glucanase to the nonredundant protein database in NCBI using BLASTP algorithm demonstrated that the AC1 Glucanase does not have significant homology to known toxic proteins. In addition, there is a long history of safe consumption of glucanase enzymes by humans and animals since glucanases are ubiquitous in plants and since they are consumed by humans and animals in the form of dietary supplements and feed additives, respectively. Application of the decision tree developed by Pariza and Johnson (2001) to determine the safety of food processing enzymes to the AC1 Glucanase demonstrated that the AC1 Glucanase is considered to be safe. Considering all of these factors, it is concluded that the AC1 Glucanase has very low potential to be a toxic protein.

Based solely on the above described information and publicly available information related to the safety of glucanase and other enzymes, a conclusion that the AC1 Glucanase is safe when consumed by animals is warranted. The conclusion that the AC1 Glucanase is safe for inclusion in animal feed at doses up to 5,000 U/kg of feed is supported by publicly available information (Broomhead *et al.*, 2019). Glucanase enzymes and other feed enzymes have been added to animal feeds for decades and to date there has never been a documented case of oral toxicity to a feed enzyme (Olemska-Beer *et al.*, 2006). The major consideration for the safety of enzyme products is the safety of the production host. The AC1 Glucanase is produced by maize that has been a key food for humans and a feed ingredient for animal feed for millennia. As such, maize is considered to be a "Safe Production Host" according to the decision tree of Pariza and Johnson (2001) and therefore safety studies for the AC1 Glucanase are not warranted, as it is extremely unlikely to be toxic. Very few of the millions of proteins found in nature are orally toxic. Those that are toxic are so due to their unique biological modes of action. In a review of enzyme safety, Ladics and Sewalt (2018) state "the weight-of-evidence indicates that there are no concerns for oral toxicity of enzymes in general, nor genotoxicity." The potential toxicity of many glucanase enzymes has been studied and they have been found to be safe for consumption by animals (EFSA, 2013a, 2013b, 2015, 2016, and 2017). Coenen *et al.* (1995) conducted a 90-day subchronic toxicity study in rats with a glucanase enzyme and found no signs of toxicity. In addition this same study showed no toxicity after feeding poultry for 28 days with a feed that was supplemented with 6,400 U/kg of a glucanase enzyme. Therefore, the collective results of many studies with different glucanase enzymes demonstrate the safety of these enzymes as a group. Since the AC1 Glucanase is primarily a glucanase that demonstrates typical glucanase biological activity the conclusion that it is safe is supported by all of the aforementioned studies that collectively demonstrate the safety of glucanase enzymes. Although the publicly available information that is currently available is sufficient to support a conclusion of the safety of the AC1 Glucanase, Agrivida, Inc. conducted and published the results of a tolerance study with poultry to further demonstrate the safety of this product. This study is described below in § 6.2.13.

6.2.13 Tolerance Study with GralNzyme® AC1 Glucanase in Chickens

A 42-day tolerance feeding study was conducted with poultry that included a high dose of GralNzyme® AC1 Glucanase equal to 5000 U/kg feed. The results and conclusions of this study are described by Broomhead *et al.* (2019). All diets used in this study conformed to the recommendations of the National Research Council (NRC, 2012). Diets were formulated to meet the NRC requirements for poultry nutrition with the exception of changes noted in Appendix 4.

Briefly, 360 chicks were divided into two treatment groups of 180 birds each, housed 15 birds to a pen. One treatment consisting of a standard age appropriate

chicken diet without supplementation with AC1 Glucanase (treatment 1, Positive control diet) and the other treatment (treatment 2) consisted of the standard diet supplemented with 5000 U/kg GraINzyme® AC1 Glucanase. A randomized complete block design was used in the experiment. Measurements taken included:

- Mortality
- Starting weights
- Feed consumption
- Body weight gains
- Feed conversion efficiency
- Hematology analysis

Summary Results the Tolerance Study

The safety of high doses of the AC1 Glucanase in broiler chickens was demonstrated by feeding chickens a feed supplemented with 5000 U glucanase/kg feed. Further details of this study have been reported by Broomhead *et al.* (2019). The ingredients and nutritional content of the feed used in this study are presented in Table 12. One batch of feed was prepared that lacked 2.78% of the feed as corn meal. This feed batch was divided into two equal aliquots and conventional corn meal was added equivalent to 2.78% of the feed (control feed) while corn meal derived from maize Event FG259 that contains the AC1 Glucanase was added to the second aliquot equivalent to 2.78% of the feed (AC1-treated feed). The feeds were pelleted in a California Pellet Mill and the pelleted feed was used in grower (15-28 days) and finisher (29-42 days) phases of the study. Birds in the starter phase (1-14 days) were fed a crumbled version of the feeds. The glucanase activity for the AC1 Glucanase supplemented starter, grower, and finisher diets was determined to be 4140, 4786, and 5020 U/kg feed, respectively.

On day 42 of the study, blood was collected from the brachial vein of three randomly selected birds per pen. A minimum of 1.0 ml of whole blood was collected in EDTA tubes for hematological analysis at a commercial lab. An additional 2.0 ml of whole blood was collected in red-top tubes and allowed to clot for 30 minutes. The tubes with clotted blood were centrifuged for 10 – 15 min at 2500 rpm and the serum was submitted to [redacted] (b) (4)) for serum biochemistry analysis. The same three birds selected for blood collection were euthanized and necropsied for pathological or toxicological symptoms. If any pathological or toxicological symptoms were noted, the tissues displaying abnormal characteristics were sampled and placed into 10% buffered formalin. These tissue samples would have been processed for histopathological evaluation, however, no pathological or toxicological symptoms were observed in the birds fed AC1-treated feed and so no histological samples were collected from the birds. The hematology and serum biochemistry results from blood samples taken on day 42 are presented in Table 13 and Table 14, respectively. No statistical differences in hematological or serum

biochemistry results between the control and AC1-treated birds were observed ($P > 0.05$).

In addition to the hematology and serum biochemistry evaluation described above, animal performance parameters were assessed. All birds were weighed by pen on days 1, 14, 28, and 42 and average body weight per pen was determined. Cumulative average bird body weight gain (BWG) was calculated through the end of each growth phase. Total pen feed intake (pFI) was calculated for each cumulative growth period by subtracting the remaining feed in the feeder (summed for each individual phase included within cumulative period; day 14, 28, and 42) from the total feed added to each pen during that period. Mortality corrected feed conversion (FCR) was calculated for each cumulative growth period by dividing pFI by mortality adjusted pen weight gain (mWG). Pen mWG was calculated by including the weight of mortality and culled bird weights for each cumulative growth period. Mortality corrected feed intake per bird (FI) was calculated by multiplying BWG by FCR. Birds consuming AC1 Glucanase-treated feed had higher cumulative FI through day 14 and day 28 ($P < 0.05$), but no significant difference was observed in FI at day 42 ($P > 0.05$) (Table 15). No statistical differences were observed in FCR, BWG or body weight between treatments during any cumulative period of the study ($P > 0.05$). In this study the overall mortality of 3.3% for control and 5.0% for AC1 Glucanase-treated groups was not statistically significant and is within expected levels based on the study conditions.

Table 12. Dietary formulations of feeds used in the tolerance study for AC1 Glucanase.

Ingredient	Starter	Grower	Finisher
	%	%	%
Corn[1]	60.22	65.65	70.57
Soybean Meal	30.49	24.94	20.50
Corn DDGS	3.00	3.00	3.00
Meat and Bone Meal	2.77	2.91	2.46
Limestone	1.12	1.08	1.06
Soybean Oil	0.71	0.97	1.06
DL-Methionine	0.39	0.34	0.30
Salt	0.37	0.40	0.41
L-Lysine	0.24	0.25	0.24
Defluorinated Phosphate	0.22	-	-
L-Threonine	0.17	0.15	0.13
Trace Mineral Premix[2]	0.10	0.10	0.10
Vitamin Premix[3]	0.10	0.10	0.10
Salinomycin[4]	0.04	0.04	-
Choline Chloride	0.04	0.04	0.04
Phytase[5]	0.02	0.02	0.02
	Calculated Nutrients		
Metabolizable Energy, kcal/kg	3,040	3113	3,166
Crude Protein, %	22.11	19.93	17.91
Calcium, %	0.96	0.88	0.82
Available Phosphorus, %	0.47	0.43	0.40
Digestible (Dig) Methionine, %	0.68	0.61	0.54
Dig Lysine, %	1.21	1.08	0.95
Dig Methionine and Cysteine, %	0.93	0.84	0.76
	Analyzed Nutrients		
Crude Protein, %	19.85	21.04	18.77
Crude Fat, %	3.67	4.13	4.06
Moisture, %	9.35	9.29	9.42
Crude Fiber, %	2.2	2.2	2.1
Ash, %	4.97	4.57	4.13

[1]AC1 corn-expressed glucanase enzyme added in place of equal portion of corn.
[2]Supplied per kilogram of feed: 88 mg of Fe (ferrous sulfate); 66 mg of Mn (manganese oxide); 44 mg of Zn (zinc oxide); 10 mg Mg (magnesium oxide); 8.8 mg Cu (copper sulfate); 0.39 mg of I (ethylenediamine dihydroiodine); 0.15 mg of Se (sodium selenite)
[3]Supplied per kilogram feed: 9,261 IU of vitamin A; 4,190 IU of vitamin D_3; 33 IU of vitamin E; 2.6 mg of vitamin K; 48 mg of niacin, 13 mg of d-pantothenic acid; 8.7 mg of riboflavin 2.9 mg of pyridoxine; 2.1 mg of thiamine; 0.95 mg of folic acid; 0.12 mg of biotin; 14 µg of vitamin B_{12}
[4]Sacox 60, 60 g/lb
[5]Supplying 500 FTU Phytase/kg Diet

Table 13. Hematology parameters[1] after feeding treatments for 42 d

Variable	Treatment		SEM	P Value
	Control	AC1[2]		
Hemoglobin, g/dL	12.11	12.14	0.20	0.92
Hematocrit, %	33.51	33.42	0.51	0.90
Red Blood Cell x10^6 uL	2.783	2.785	0.037	0.98
Mean Corpuscular volume, fL	120.4	120.1	0.6	0.71
MCH, pg[3]	43.52	43.61	0.23	0.78
MCH concentration, g/dL	36.15	36.32	0.11	0.30
Red Cell Distribution Width, %	9.488	9.248	0.125	0.20
White Blood Cell x10^3 ul	16.95	21.43	2.62	0.25
Heterophils, %	42.31	35.56	3.01	0.14
Lymphocytes, %	43.75	48.94	2.71	0.20
Monocytes, %	4.666	4.277	0.320	0.41
Eosinophil, %	6.098	6.694	1.020	0.69
Basophil, %	4.500	4.624	0.354	0.81
Absolute Heterophils, x10^3 ul	6.169	7.203	0.729	0.34
Absolute Lymphocytes, x10^3 ul	8.24	11.24	1.68	0.23
Absolute Monocytes, x10^3 ul	0.765	0.780	0.101	0.48
Absolute Eosinophil, x10^3 ul	1.178	1.280	0.257	0.78
Absolute Basophil, x10^3 ul	0.883	0.857	0.138	0.90

[1]Average of three birds per replicate pen and 12 pens per treatment.
[2]AC1 = AC1 Glucanase added to Control diet
[3]MCH = Mean cell hemoglobin

Table 14. Serum biochemistry parameters[1] after feeding treatments for 42 d.

Variable	Treatment		SEM	P Value
	Control	AC1		
Total Protein, g/dL	3.142	3.100	0.053	0.59
Albumin, g/dL[3]	1.142	1.126	0.019	0.56
Globulin, g/dL	2.014	1.990	0.038	0.66
Albumin/Globulin	0.571	0.565	0.010	0.69
Creatine Kinase, U/L[3]	40,465	44,267	2761	0.35
Alanine Aminotransferase, U/L[5]	-	-	-	-
Phosphorus, mg/dL	6.848	6.905	0.062	0.52*
Glucose, mg/dL	245.3	249.9	1.7	0.09*

[1]Average of three birds per replicate pen and 12 pens per treatment.
[2]Four out of 72 samples were below analyzable limits (<1.0 g/dL).
[3]Unofficial values; 63 out of 72 samples were above the accurately analyzable limits (>22500 U/L) and were estimated by lab.
[4]63 out of 72 samples were below analyzable limits (<5 U/L) and non-estimable.
*Statistical significant blocking effect was observed (P < 0.05).
AC1 = AC1 Glucanase added to Control diet

Table 15. Intermittent and overall cumulative animal performance[1]

Treatment	Feed Intake, kg	Body Weight Gain, kg	Feed Conversion, kg:kg	Body Weight, kg[2]
1 – 14 d Performance				
Control	0.297[b]	0.239	1.246	0.278
AC1	0.309[a]	0.251	1.230	0.290
SEM	0.004	0.004	0.009	0.004
P Value	0.047	0.062	0.247	0.065
1 – 28 d Performance				
Control	1.600[b]	1.192	1.343	1.231
AC1	1.662[a]	1.231	1.351	1.269
SEM	0.018	0.019	0.009	0.019
P Value	0.037	0.183	0.568	0.185
1 – 42 d Performance				
Control	4.120	2.669	1.545	2.708
AC1	4.200	2.712	1.549	2.751
SEM	0.042	0.038	0.009	0.038
P Value	0.204*	0.440	0.724	0.444

[1]Mortality corrected performance; average of 12 replicate pens with 15 birds per pen.
[2]Ending period body weight.
*Statistically significant blocking effect was observed ($P \leq 0.05$).
AC1 = AC1 Glucanase added to Control diet

Discussion of Results of the Tolerance Study

The absence of significant changes in key hematological parameters and of indicators of toxicity or abnormalities in the tissues of the birds in the study that received a diet treated with 5000 U/kg AC1 Glucanase support a conclusion that high doses of the AC1 Glucanase up to 5000 U/kg feed are well tolerated by broiler chickens and are safe. This conclusion is further supported by the good performance of the chickens in the AC1-treated group as demonstrated by body weight gain and feed conversion rates that are summarized above. These results demonstrating the safety and tolerance of chickens to high doses of the AC1 Glucanase are consistent with results reported for a similar study in which broiler chickens were fed a diet supplemented with 6400 U/kg of a β-glucanase enzyme for 28 days without any indications of toxicity or negative impacts on hematology indicators or animal performance (Coenen *et al.*, 1995).

6.2.14 Conclusions of the Safety of GralNzyme® AC1 Glucanase

The biological activity of the GralNzyme® AC1 Glucanase has been well characterized (§6.2.8) and was demonstrated to be primarily a β-1-4 glucanase with

minor levels of other carbohydrase activities. These activities are known to be safe, and similar enzymes are present in commonly consumed food and in human nutritional supplement products. A decision tree designed to assess the safety of enzymes used in food preparation [Pariza and Johnson, 2001] was applied to the AC1 Glucanase produced by Event FG259 and the results indicated that the AC1 Glucanase should be considered safe for food use. A bioinformatic assessment of the amino acid sequence of the AC1 Glucanase protein revealed that it does not share significant homology with known protein toxins (§6.2.10). The tolerance study in which chickens were grown for 42 days on diets containing 5000 U GralNzyme® AC1 Glucanase/kg of feed described in §6.2.13 and Broomhead *et al.* (2019) demonstrates that the growth and weight gain of the treated chickens was not different from that of the control group whose feed did not contain glucanase. Furthermore, the results of hematological analyses of the chickens at the end of the study (42 days) confirms that there were no differences between the GralNzyme® AC1 Glucanase treated and control groups that would indicate that the high dose of GralNzyme® AC1 Glucanase in the treated group resulted in any negative safety issues. In the course of the necropsies performed on the chickens from both the GralNzyme® AC1 Glucanase treated and control groups there were no indications of health or safety issues in tissues of the treated group relative to the controls. The results of studies conducted by Agrivida, Inc. described herein support a conclusion that the inclusion of GralNzyme® AC1 Glucanase in the feed of chickens at up to 5,000 U/kg is safe and effective and does not impede the growth or normal development of the chickens.

6.3 Enzyme Functionality in Poultry

Feed grains such as rye, barley, oats and wheat contain high amounts of soluble NSP that result in increased viscosity of digesta in animals that consume feeds based on these grains. The increased viscosity of digesta in the GI tracts of monograstric animals results in reduced nutrient digestibility and availability and negative impacts on the gut microbiome (Burnett, 1966; Choct and Annison, 1992; Bedford and Classen, 1992; Danicke et al., 1999). Since the 1980s glucanase and other carbohydrase enzymes that degrade soluble NSP and reduce the viscosity of digesta in the GI tract have been added to the feed of monogastric animals to increase performance of animals fed diets based on grains with a high soluble NSP content (Hesselman and Åman, 1986; Campbell *et al.*, 1989; Broz and Frigg, 1986; Newman and Newman, 1987). The functionality of the AC1 Glucanase in poultry has been demonstrated to cause a reduction in the viscosity of the digesta of broiler chickens in a poultry feeding trial that was conducted at (b) (4) (Ayres *et al.*, 2018). The AC1 Glucanase was also shown to reduce the viscosity of feed that included wheat and dried distiller's grain solids (DDGS) *in vitro* (Ayres *et al.*, 2019). The positive effects on the performance of broiler chickens provide further support of the functionality of the AC1 Glucanase when it was included in the feed (Ayres *et al.*, 2018 and Jacela *et al.*, 2018).

6.3.1 Digesta Viscosity: Summary of the results from a broiler chicken study

A commercial breed of Hubbard x Cobb 500 chickens were grown for 21 days on a standard corn/soybean meal diet treated with four levels of AC1 Glucanase (0, 50, 100, 200 and 400 U/kg). The diet of the birds in these treatment groups included 10% wheat and DDGS (Table 16). A positive control group was fed a feed without wheat or DDGS (Table 16). On day 14 of the study, four birds from each pen were euthanized by cervical dislocation and the contents of the entire digestive tract were collected. The viscosity of the digesta was measured and is presented in Table 17.

As illustrated in Table 17, birds fed a corn/soybean diet (PC) had the lowest digesta viscosity, which was statistically significantly lower than those receiving the negative control (NC) basal diet that included wheat and DDGS. This demonstrates that the inclusion of wheat and DDGS in the diet results in increased viscosity of the digesta. The viscosity of the digesta decreased with increasing dose of the AC1 Glucanase except for one treatment (NC1+100 U/kg), where the viscosity was numerically higher than NC treatment. These results demonstrate the functionality of the AC1 Glucanase by its ability to reduce the viscosity of the digesta in the digestive tracts of birds fed a diet treated with it. A more detailed report of this study was published by Ayres *et al.* (2018).

Table 16. Diet formulations of a positive control (corn/soybean) diet and a negative control diet amended with wheat and DDGS.

Ingredients	Inclusion (%)	
	Positive Control[1]	Negative Control
Corn	59.92	48.36
Soybean Meal	32.47	25.84
Wheat	--	10
DDGS	--	10
Dicalcium Phosphate	1.82	1.74
Limestone	1.35	1.38
L-Lysine	0.29	0.43
DL-Methionine	0.35	0.34
Soybean Oil	2.93	1.11
Vit/Min Premix	0.25	0.25
L-Threonine	0.19	0.23
White Salt	0.33	0.21
Sodium Bicarbonate	0.10	0.10
Calculated Nutrients[2]		
ME (kcal/kg)	3,000	2,900
Crude Protein (%)	20.00	20.00
Calcium (%)	1.01	1.01
Available Phosphorus (%)	0.46	0.46
Dig Methionine (%)	0.63	0.62
Dig Lysine (%)	1.20	1.20
Dig Methionine and Cystine (%)	0.90	0.90

[1]The positive control diet contained 100 kcal/kg ME more than the negative control diets.
[2] Metabolizable Energy and Available Phosphorus were based on Agristat values as suggested by M. Donohue. 2013. A 2.2 ratio was maintained for Ca to AP. A 4.4 Ca to AP ratio was created for the negative control. Digestible amino acids were based on the digestible lysine value (1.2%) suggested by Tillman and Dozier (2014). Digestible amino acid to digestible lysine ratios followed further recommendations of this communication (47 methionine, 77 TSAA, 68 threonine, 16 tryptophan).

Table 17. Digesta viscosity of broilers at day 14 that were fed a corn, soybean meal, wheat, and DDGS diet supplemented with AC1 Glucanase. The composition of the diets is presented in Table 16. Values listed within each category that share the same statistical letter designation are not significantly different at a P-value <0.05.

Diet Formulation (AC1 Glucanase U/kg feed)	AC1 Glucanase activity recovered from feed	Digesta Viscosity (cP)
	U/kg	10 X g
		1 min
NC + 0	-	3.55[ab]
NC + 50	29.4 ± 7.7	3.36[ab]
NC + 100	90.3 ± 13.6	3.78[a]
NC + 200	270.6 ± 26.6	3.23[bc]
NC + 400	609.9 ± 48.9	3.20[bc]
Positive Control[1]	-	2.86[c]
Treatment P-value		0.0056
Treatment SEM[2]		0.163
Fisher's LSD[3]		0.463

[1]The positive control diet contained 100 kcal/kg ME more than the negative control diets.
[2]SEM= Standard Error of the Mean
[3]Fisher's least significant difference multiple comparison test (a, b, c)
[a-c]Means with superscripts without a common letter differ significantly (P < 0.05).

6.3.2 Reduction of the viscosity of feed

An *in vitro* study of the ability of the AC1 Glucanase to reduce the viscosity of feed was also conducted at (b) (4). Briefly, eight feeds based on corn/soybean meal/wheat/DDGS were prepared that included positive and negative controls and five levels of inclusion of the AC1 Glucanase (Table 18). The formulation of these feeds is representative of commercial diets that include wheat and DDGS and is presented in Table 18. The different feed treatments included the following:

1. Positive control 1 (PC_1): corn/soybean, ME 3050 kcal/kg
2. Positive control 2 (PC_2): corn/soybean mixed with 10% wheat and 10% DDGS, ME 3050 kcal/kg
3. Negative control (NC): corn/soybean mixed with 10% wheat and 10% DDGS, ME 2925 kcal/kg
4. NC + 50 U AC1 Glucanase/kg
5. NC + 100 U AC1 Glucanase/kg
6. NC + 200 U AC1 Glucanase/kg
7. NC + 400 U AC1 Glucanase/kg
8. NC + 600 U AC1 Glucanase/kg

A sample of each of the feed preparations was ground with an air-assisted hammer mill. The viscosity of the ground samples was assessed in gastric simulated digestion solutions as described by Bedford and Classen (1993). Briefly, 0.6 g ground sample was weighted in duplicate and mixed with a 0.1 *N* HCl solution (1.8 ml) containing 2,000 U pepsin/mg, and incubated for 45 min with occasional vortexing at 40°C. To simulate pancreatic digestion, 0.6 ml of a 1 *M* $NaCO_3$ solution containing 2 mg/ml pancreatin (8 x USP) was added to the mixture. The mixture was incubated at 40°C for 120 min with occasional vortexing. The contents were removed and placed in microcentrifuge tubes and centrifuged at 12,700 x g for 2 min. About 1 ml of supernatant was transferred into a second microcentrifuge tube and then placed in a 25°C water bath for approximately 10 minutes. A total 0.5 mL of supernatant was placed into a Brookfield cone and plate viscometer utilizing a CPE-40 cone and CPE-44Y cup. Viscosity measurements were made at 30 s and 1 min at two speeds of 10 x g and 20 x g.

The effect of the inclusion of AC1 Glucanase in dietary viscosity at different rates is presented in Table 19. The dietary viscosity produced with the PC1 feed that did not contain wheat or DDGS was lower (1.81 cP) compared to the PC2 feed (2.20 cP) and the NC feed (1.87 cP) that contained 10% wheat and DDGS (Table 19). This result confirms that the addition of wheat and DDGS to the feeds resulted an increase in dietary viscosity. Feeds with AC1 Glucanase inclusion prepared by the gastric simulated digestion had viscosities that were lower compared to those prepared from the PC2 and NC feeds that included wheat and DDGS but did not contain AC1 Glucanase (Table 19). There was a general trend in which feeds with higher amounts of AC1 Glucanase tended to have lower viscosity. The NC feeds with

50 and 100 U AC1 Glucanase/kg feed generated viscosities that were generally equivalent to each other (1.82 and 1.83 cP, respectively) but were lower than that of the NC feed (1.87 cP) (Table 19). The higher level of AC1 Glucanase in the NC + 200 U feed resulted in a further decrease of viscosity to 1.74 cP. The digested feed with the highest amounts of AC1 Glucanase, 400 and 600 U/kg, generated the lowest viscosities in the experiment (1.66 and 1.72 cP, respectively) (Table 19). These results are consistent with those from the *in vivo* animal study described in §6.3.1 above in which it was demonstrated that the inclusion of AC1 Glucanase in the feed of chickens results in a reduction of the viscosity of the intestinal digesta and provides further confirmation of the intended functionality of AC1 Glucanase in feed. Details of this study have been reported by Aymes *et al.* (2019).

Table 18. Feed formulations used in the *in vitro* study of the impact of AC1 Glucanase on dietary viscosity.

Ingredients	Inclusion (%)		
	Positive Control Corn-Soy[1]	Positive Control Wheat and DDGS[1]	Negative Control
Corn	53.02	37.83	40.24
Soybean Meal	37.83	33.08	32.77
Wheat	---	10	10
DDGS	---	10	10
Dicalciutn Phosphate	1.79	1.71	1.7
Limestone	1.32	1.36	1.37
L-Lysine	0.13	0.22	0.22
DL-Methionine	0.49	0.48	0.47
Soybean Oil	4.44	4.44	2.35
Vit/Min Premix	0.25	0.25	0.25
L-Threonine	0.30	0.31	0.31
White Salt	0.33	0.22	0.22
Sodium Bicarbonate	0.10	0.10	0.10
Calculated Nutrients[2]			
ME (kcal/kg)	3,050	3,050	2,925
Crude Protein (%)	22.25	22.86	22.86
Calcium (%)	1.01	1.01	1.01
Available Phosphorus (%)	0.46	0.46	0.46
Sodium (%)	0.17	0.17	0.17
Dig Lysine (%)	1.20	1.20	1.20
Dig Methionine (%)	0.79	0.78	0.77
Dig Methionine and Cysteine (%)	1.08	1.09	1.08
Threonine (%)	1.01	1.01	1.01
Tryptophan (%)	0.24	0.24	0.24
Analyzed Nutrients			
Crude Protein (%)	22.9	21.4	21.65
Crude Fat (%)	5.34	6.37	4.81

[1] The positive control diets contained 125 kcal/kg ME more than the negative control diets.
[2] Metabolizable Energy and Available Phosphorus were based on Agristat values as suggested by Donohue (2013). Digestible amino acids were based on the digestible lysine value (1.2%) suggested by Tillman and Dozier (2013). Digestible amino acid to digestible lysine ratios followed further recommendations of this communication (minimum of 0.54 methionine, 1.02 TSAA, 0.90 threonine, 0.21 tryptophan).

Table 19. *In vitro* viscosity test on feed containing different inclusion rates of the AC1 Glucanase.

Diet Formulation	Viscosity (cP) 10 x g - 30s
PC_1: Corn-SBM[1]	1.81
PC_2: Wheat and DDGS[2]	2.02
Negative Control (NC)[3]	1.87
NC + 50U AC1	1.82
NC + 100U AC1	1.83
NC + 200U AC1	1.74
NC + 400U AC1	1.66
NC + 600U AC1	1.72

[1] Positive control diet composed corn and soybean meal
[2] Positive control based diet including 10% wheat and 10% DDGS
[3] Similar to PC_2 but with ME reduced by 125 kcal/kg.

6.3.3 Positive impact of AC1 Glucanase on poultry performance

The functionality of the AC1 Glucanase in poultry feed is further supported by the demonstration that its inclusion in feed has a positive impact on various aspects of animal performance. Ayres *et al.* (2018) report that the inclusion of AC1 Glucanase in a high-NSP diet at 400 U/kg increased the weight gain after 14 days to be equivalent to that of the animals that received the low-NSP PC diet. It was also demonstrated in this study that the FCR of the birds receiving the high-NSP diet with 400 U/kg AC1 Glucanase was equivalent to that of the control group receiving the low-NSP diet without AC1 Glucanase.

In a similar study, Jacek *et al.* (2018) reported a positive impact on animal performance criteria due to the inclusion of AC1 Glucanase in the feed. In this study 728 Cobb 500 male broilers were randomly assigned to seven treatments with 13 replicates per treatment. The treatments consisted of a PC that received a standard diet, a NC that was formulated to contain 132 kcal less energy than the PC diet, and five treatments that received the NC diet amended with either 5, 50, 100, 250, or 500 U/kg AC1 Glucanase. After 16 days, measurement of body weight, FCR, and ileal digestibility of energy (IDE) measurements were made. Jacek et al. (2018) reported that inclusion of the AC1 Glucanase at 100 and 250 U/kg resulted in a significant increase ($p<0.01$) in body weights compared to both the PC and NC groups. In addition, the inclusion of AC1 Glucanase to the NC diets at more than 100 U/kg resulted in a significant increase ($p<0.01$) in IDE and restored the IDE to be equivalent to that of birds that received the PC diet ($p<0.05$). These results are consistent with those reported by Ayres *et al.* (2018) and they provide further support of the functionality of the AC1 Glucanase in poultry diets.

6.3.4 Conclusions on the Functionality of GralNzyme® AC1 Glucanase

The ability of the AC1 Glucanase to decrease the viscosity of the digesta in the GI tract of poultry when included in feeds that contain wheat and DDGS has been demonstrated by Ayres *et al.* (2018). This finding is supported by another study (Ayres et al., 2019) where the viscosity of wheat and DDGS containing feed were reduced by the addition of the AC1 Glucanase. Additional support of the functionality of the AC1 Glucanse in poultry feeds comes from two studies, one reported by Jacek *et al.* (2018) in which the inclusion of the AC1 Glucanase in poultry feed was demonstrated to increase body weight and IDE and to improve FCR. A second study by Ayres *et al.* (2018) demonstrated improved weight gain and FCR in broiler chickens receiving a feed supplemented with AC1 Glucanase. Taken together, these studies provide clear confirmation of the functionality of the AC1 GralNzyme® Glucanase in the feed of poultry.

6.3.5 Safety of human consumption of meat produced by animals treated with GralNzyme® AC1 Glucanase

The meat derived from animals that consume feed treated with GralNzyme® AC1 Glucanase is safe for human consumption and does not present any human safety concerns. The GralNzyme® AC1 Glucanase is an enzyme and enzymes are proteins. The dietary fate of the GralNzyme® AC1 Glucanase in animals that consume feed treated with it is the same as that of all other proteins in the animal's diet that are digested into the constituent amino acids of the dietary proteins (Metcalf *et al.*, 1996; Betz *et al.*, 2000). As part of an Early Food Safety Evaluation for the GralNzyme® AC1 Glucanase that was submitted to the U.S. FDA Center for Food Safety and Nutrition, Agrivida, Inc. demonstrated that the GralNzyme® AC1 Glucanase enzymie is sensitive to digestion in a simulated gastric environment (FDA/CFSAN, 2018). Therefore, the GralNzyme® AC1 Glucanase is expected to be digested in the gastrointestinal tracts of animals and is not expected to be absorbed intact into the blood of animals that consume it, nor to be deposited into the tissues of the animals, including the meat. The safety of glucanase feed additives for humans that consume meat from animals that consume feed treated with glucanases is further supported by the fact that glucanases have been included in the feed of poultry for decades without any adverse effects on human health or nutrition.

6.4 JECFA Specifications

Each of the three representative AC1 Glucanase product batches that are described in §6.2.2 were analyzed to demonstrate that they meet the purity, chemical and microbial specifications established for enzyme preparations, as outlined in the specifications established for enzymes used in food processing, as proposed by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2006). Physical,

chemical, and microbial characteristics were determined for each of the AC1 Glucanase product batches by [redacted] (b) (4) [redacted] (b) (6). The results of these analyses are presented in Table 20.

Examination of the results of the analysis of key product characteristics as presented in Table 20 demonstrate that all three AC1 Glucanase product batches meet or exceed all JECFA specifications established for enzyme preparations that are used in food and/or feed with the exception of total bacterial count and the number of coliform colony forming units (cfu). All three product batches had no detectible presence of either *Salmonella* or *E. coli* bacteria. Coliform bacteria are defined as rod-shaped Gram negative, non-spore forming and motile or non-motile bacteria that can ferment lactose with the production of acid and gas when incubated at 35-37°C (Brenner, 1992; Bettelheim, 1992). While coliforms themselves are not normally causes of serious illness, their presence has been used to indicate that other pathogenic organisms of fecal origin may be present (Krentz et al., 2013). Typical genera in the coliform group include: *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, and *Escherichia* (Brenner, 1992; Bettelheim, 1992).

The JECFA specifications for food enzyme preparations have been traditionally applied to enzyme products that are produced by axenic fermentation followed by purification of the enzyme in a sanitary laboratory environment. Under these conditions it is feasible to produce a purified enzyme product that meets the JECFA specifications for the presence of microbes in the product. However, the AC1 Glucanase product is produced in the same manner as the production of maize grain that is widely used as a major component of human food and animal feed. It is produced in agricultural fields in the environment where bacteria are present in the soil, air and water and on the surfaces of plants, including the maize that produces the AC1 Glucanase-containing grain. Therefore, it is reasonable to expect that the AC1 Glucanase product would contain levels of bacterial presence that are typical for maize grain produced by conventional agricultural practices. All of the three AC1 Glucanase product batches exceeded the JECFA specification of 30 cfu/g product for coliform bacteria with coliform numbers of 3,700 to 13,000 cfu/g (Table 20). However, this range is consistent with studies of microbial presence in maize grain and in animal feed. Tabib et al. (1981) surveyed feeds and feed ingredients, including maize, in the feed of broilers, layers and turkeys and found that the numbers of coliform bacteria ranged from 450 – 910,000 cfu/g. Similar studies have also reported equivalent levels of coliform bacterial in cattle feed (Sanderson *et al.*, 2005) and tortillas made from corn meal (Gomez-Aldapa *et al.*, 2013). From these reports it is evident that the level of coliform bacteria in AC1 Glucanase product batches is similar to those reported as normal for maize grain and other commonly used feed ingredients. Since the numbers of coliform bacteria found to be present in AC1 Glucanase product batches are typical for those found in maize grain and other animal feed ingredients and since known pathogenic bacteria such as *Salmonella* and *E. coli* were absent from the product batches, the higher level of

coliforms in the AC1 product compared to the JECFA specifications for food enzyme products is considered to be safe.

Table 20. Physical, chemical, and microbial characteristics of three independent AC1 product batches compared to JECFA specifications for enzyme preparations used in food and feed.

			Glucanase AC1 Product Batch			
Characteristics	Method	Unit	AV_AC1_0070	AV_AC1_0075	AV_AC1_0077	JECFA Specification Limit
Physical						
AC1 Activity	Agrivida, Inc. SOP	U/g	(b) (4)			NA
	Agrivida, Inc. SOP	U/mg protein			(b) (4)	NA
Ash	AOAC 942.05	%	1.24	1.62	1.17	NA
Density	USP 616	g/ml	0.5	0.5	0.6	NA
Micron particle size	MF-2051 Evaluating Particle Size, KSU 2002		Listed	Listed	Listed	NA
Chemical						
Cadmium	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	≤0.010	≤0.010	≤0.010	30 max
Mercury	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	≤0.010	≤0.010	≤0.010	30 max
Lead	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	≤0.010	≤0.010	≤0.010	5 max
Arsenic	J. AOAC vol. 90 (2007) 844-856 (Mod)	mg/kg	≤0.010	≤0.010	≤0.010	3 max
Microbial						
Coliforms	AOAC 991.14	cfu/g	3700	11000	13000	30 max
Salmonella	AOAC 2003.09	#/25g	Not Detected	Not Detected	Not Detected	Absent
Aerobic Plate Count	FDA BAM Chapter 3	cfu/g	16000	61,000 (est)	66,000 (est)	50,000 max
Escherichia Coli	U.S. Pharmacopeia Chapter 62	#/10 g	Not Detected	Not Detected	Not Detected	Absent
Aflatoxin	Commercial Test Kit (ELISA)	ppb	≤5	≤5	≤5	ND*
T-2/HT-2 Toxin	Commercial Test Kit (ELISA)	ppb	≤25	≤25	≤25	ND
Ochratoxin	Commercial Test Kit (ELISA)	ppb	≤2	≤2	≤2	ND
Sterigmatocystin	(b) (4)	Pg/kg	≤10	≤10	≤10	ND

6.5 Stability and Homogeneity of the AC1 Glucanase Product

The stability of AC1 activity in three representative AC1 product batches over time and under different storage conditions was examined. Three representative AC1 product batches were produced (Batches AV_AC1_00070, AV_AC1_00074 and AV_AC1_00077) and were characterized as described in Appendix 2.

6.5.1 Product Stability

Four grams of dry product material from each product batch was put into small, double paper pouches made from larger seed-type bags. The openings of the sample bags were sewn closed. Sample packages were stored at three different temperatures, i.e. refrigerated (5°C), ambient (25°C/60% RH) and accelerated (40°C/75% RH). At the initiation of the study two sample packages of each batch were opened and the contents of each were milled in a Cyclotech grinder to a particle size of less than 0.5 mm. Two 0.5g aliquots from the milled material of each sample were extracted in AC1 Glucanase assay buffer and analyzed for glucanase activity. The results of 4 analyses set the baseline of each batch for the AC1 Glucanase activity in the samples and were used as the starting activity for the storage stability study. The remaining product sample packages of each batch were separated into three groups and placed in storage under refrigerated, ambient, and accelerated conditions. As a reference control, AC1 Glucanase protein was purified from Event FG259 grain and diluted to 50 mg/ml in storage buffer (40% glycerol, 50mM MES, 150mM sodium chloride, pH 6.3) and stored under refrigerated conditions. When used as a positive control to validate the assay, the AC1 Glucanase control was diluted 100-fold and 50 µl of this dilution was added to assay buffer in a final volume of 0.5 ml. At each sampling time, one sample package of each batch was removed from each of the three storage conditions at 1, 2, 3, 6, 9, 12, 18, and 24 months after initiation of the study, and three 0.5g aliquots from the milled material of each sample were extracted in AC1 Glucanase assay buffer and analyzed for glucanase activity to generate 27 analyses at each time point. The averages of the 3 analyses for each sample are presented in Table 21. The product stability study was conducted by (b) (4).

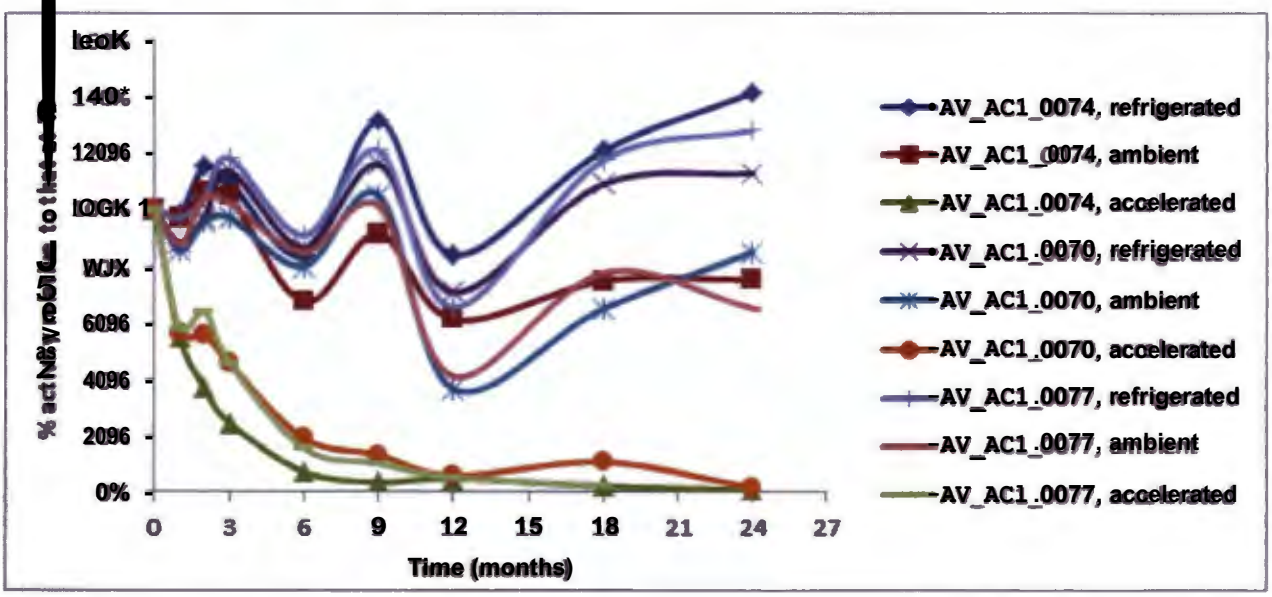
The results of the product stability study that measured the glucanase activity of three AC1 Glucanase product batches were reported as A590 absorbance units/mg from (b) (4), and are presented in Table 21 as unit/g after converting the A590/mg measurement, as described in Appendix 3. Reference activity is presented as unit/mg protein. These results demonstrate that the glucanase activity of all three AC1 Glucanase product batches stored under refrigerated or ambient conditions maintained their original glucanase activity, illustrating that the glucanase activity in the AC1 Glucanase product is stable for up to 24 months under either refrigerated conditions and up to 9 months under ambient storage conditions. The AC1 Glucanase maintained 65-85% of its activity

after 24 months of storage under ambient conditions. Likewise, the purified AC1 Glucanase protein stored under refrigerated condition maintained activity for the duration of the 24 month period. On the other hand, the AC1 product samples stored under accelerated conditions demonstrated a reduction of glucanase activity after 2 months and retained from 2 to 11% and only 2% of their initial activity after 18 and 24 months of storage, respectively (Figure 16).

Table 21. Glucanase product stability study during 24 months of storage under refrigerated (5°C), ambient (25°C and 60% relative humidity), and accelerated (40°C and 75% relative humidity) storage conditions. The results presented are in units of glucanase activity/g of product and are averages of triplicate assays at each storage condition and each sampling time point for each sample. The purified AC1 Glucanase protein stored under refrigerated condition is listed as Reference sample.

Sample	Storage conditions	Months								
		T0	1	2	3	6	9	12	18	24
AV_AC1_0074	Refrigerated	294.3	289.8	339.3	323.1	247.5	387.0	247.5	355.5	416.7
	Ambient	294.3	286.2	313.2	307.8	198.9	269.1	180.0	221.4	222.3
	Accelerated	294.3	162.0	109.8	72.9	21.6	10.8	14.4	6.3	2.7
AV_AC1_0070	Refrigerated	257.0	226.8	249.3	291.6	222.3	297.9	182.7	279.9	289.8
	Ambient	257.0	219.6	245.7	249.3	206.1	270.0	94.5	167.4	218.7
	Accelerated	257.0	144.9	143.1	117.9	49.5	34.2	15.3	27.9	4.5
AV_AC1_0077	Refrigerated	253.8	245.7	263.7	299.7	230.4	306.9	166.5	297.9	324.9
	Ambient	253.8	223.2	264.6	259.2	216.0	256.5	104.4	198.0	165.6
	Accelerated	253.8	147.6	162.9	117.0	39.6	26.1	13.5	6.3	1.8
Reference	Refrigerated	236.4	198.0	254.6	273.5	221.1	245.3	211.9	273.5	226.2

Figure 16. Percentage of AC1 Glucanase activity in the samples of AC1 Glucanase product at monthly intervals relative to the initial activity at T0 after storage under three different temperatures up to 24 months.



6.5.2 Stability of the AC1 Glucanase in feed mixtures

In order to investigate the stability of AC1 Glucanase in feed mixtures at different storage conditions, two studies of in-feed stability were conducted. Both studies (Study 1 & Study 2) used typical corn/soybean meal-based poultry feed and were prepared and mixed with a target dose of 100 U/kg at (b) (4) and Agrivida, Inc. (Study 1 and 2, respectively). Feed in Study 1 containing AC1 Glucanase product was pelleted at 80°C at (b) (4) Feed in Study 2 containing AC1 Glucanase product was pelleted at 70°C at Agrivida, Inc. One kilogram of manufactured feed from each study was put into double heavy paper pouches made from large seed bags. The opening of the bags were sewn closed to seal. The packaged feed samples were shipped to (b) (4) ((b) (6)).

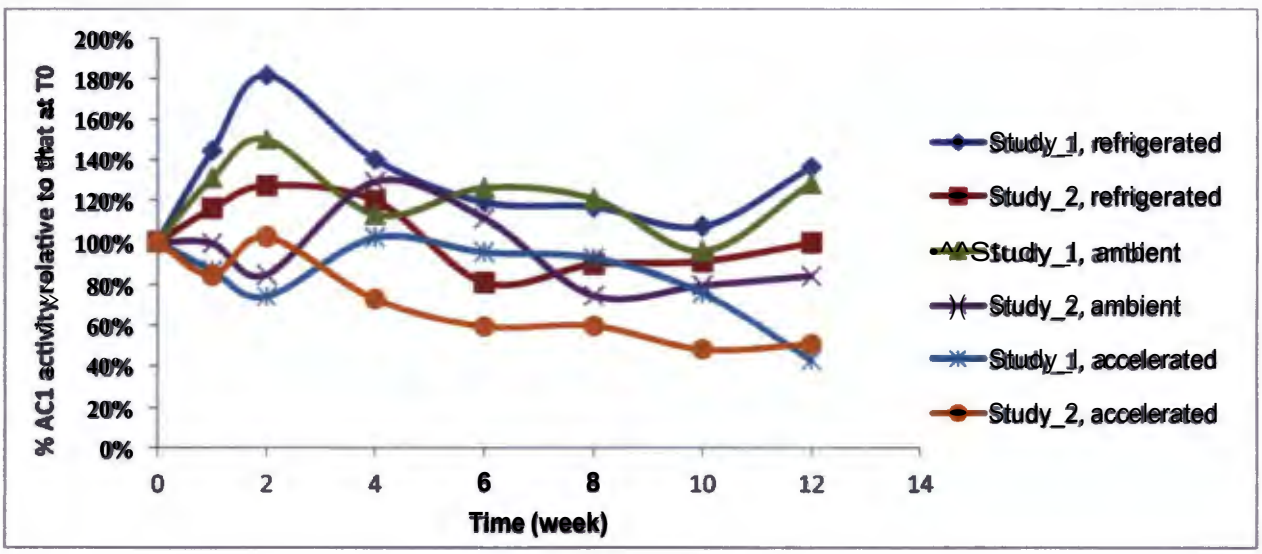
The AC1 in-feed samples were divided into three groups that were stored under refrigerated, ambient, or accelerated conditions in the same manner as described in the AC1 product stability study. As a reference control,the purified AC1 protein reference described in the product stability study was used. At various time points during the study one package of the feed mixture from each storage condition was removed and 250g of the feed mixture was ground as described in the AC1 product stability study. Three 20 g aliquots of the ground feed sample from each package were extracted with buffer and were analyzed for AC1 activity to generate three activity determinations per sample.

The average glucanase activity values from the triplicate assays of each sample stored up to 12 weeks are shown in Table 22 and Figure 17. All feed samples contained approximately 100 U/kg of AC1 Glucanase at the initiation of the study. After 12 weeks of storage the samples stored under refrigerated and ambient conditions retained more than 84% of the original activity at the start of the study. These results demonstrate that AC1 Glucanase in feed mixtures retains its activity when stored for up to 12 weeks under refrigerated or ambient conditions. Likewise, the purified AC1 Glucanase protein stored under refrigerated conditions maintained activity for the duration of the 12 weeks period. On the other hand, the glucanase activity in feed mixtures stored under accelerated conditions demonstrated a steady decline and retained approximately 43% and 51% of the original activity after 12 weeks of storage, respectively.

Table 22. Glucanase activity in feed mixtures containing AC1 Glucanase product after 12 weeks of storage under refrigerated (5°C), ambient (25°C and 60% relative humidity), and accelerated (40°C and 75% relative humidity) conditions. The results presented are U/kg glucanase activity in feed and are averages of triplicate assays at each storage condition and each sampling time point for each sample. The data from two studies are included. The purified AC1 Glucanase protein stored under refrigerated condition is listed as Reference (Ref) sample and its activity is present as U/mg protein.

Study ID	Storage Temp (°C)	Weeks							
		T0	1	2	4	6	8	10	12
1	Refrigerated	90.7	130.8	164.7	126.9	108.0	105.9	97.5	123.3
	Ambient		118.5	135.9	102.6	114.3	109.5	87.0	116.1
	Accelerated		78.6	67.5	92.7	86.4	84.0	69.0	38.7
2	Refrigerated	105.1	121.8	133.5	126.0	85.2	94.2	95.7	104.7
	Ambient		104.4	89.1	135.6	117.0	78.6	83.7	88.5
	Accelerated		88.8	107.7	76.8	62.4	62.7	50.4	53.1
Ref	Refrigerated	236.4	209.3	219.4	216.4	214.4	222.1	205.9	239.9

Figure 17. Percentage of AC1 activity in feed mixtures containing Glucanase AC1 at weekly intervals after up to 12 weeks of storage under three different temperatures relative to the corresponding activity of each sample at T0. Results from Study 1 and 2 are presented.



6.5.3 Homogeneity of AC1 Glucanase in feed mixtures

A study on the homogeneity of the AC1 Glucanase product in standard corn/soybean meal based poultry diets was conducted at (b) (4) and is reported by Ayres *et al.* (2018). A 1,225 kg batch of feed was mixed in a vertical mixer with 4.232 kg of AC1 Glucanase product that had an activity of 145 unit/g to make a diet with a target dose of 500 unit/kg diet. Ten 0.5 kg samples were collected randomly from the mixed feed and the β-glucanase activity was measured. The results of the analyses of glucanase activity of the 10 samples of mixed feed are presented in Table 23. The average glucanase activity of all samples was 425 unit/kg with a coefficient of variation (CV) of approximately 6.22% (Table 23).

Table 23. Glucanase activity of 10 randomly collected samples of feed produced to include the AC1 Glucanase product with a target dose of 500 U/kg. The activity of each replicate mash diet was the mean of two assays from the same protein extract (from Ayres *et al.*, 2018).

Replicate	Activity (U/kg)
1	411.95
2	394.10
3	471.05
4	401.30
5	433.70
6	428.15
7	448.85
8	392.00
9	451.85
10	420.20
Mean	425.32
SD	26.44
CV	6.22%

A second study on the homogeneity of AC1 Glucanase product in poultry diets made with corn/soybean was conducted at (b) (4) and was reported by Jacek *et al.* (2018). AC1 Glucanase product with an activity of 170 U/g was mixed into 1500 lbs of feed to achieve a 100 U/kg target dose. Ten 500g samples were collected randomly from the mixed feed and were subjected to glucanase activity analysis. The results of the glucanase activity analysis of the 10 samples of mixed feed with a target dose of 100 U/kg are presented in Table 24. The average glucanase activity in the feed samples was 97.24 U/kg with a CV of approximately 9.98% (Table 24).

Table 24. Results of Glucanase AC1 activity of 10 randomly collected samples of feed produced with a target dose of 100 unit/kg AC1. The activity of each replicate mash diet was an average of two assays from the same protein extract (from Jacek *et al.*, 2018).

Replicates	Activity (U/kg)
1	103.16
2	109.62
3	97.37
4	106.46
5	103.58
6	79.33
7	83.85
8	99.59
9	97.45
10	91.97
Mean	97.24
SD	9.70
CV	9.98

In summary, enzyme activity analysis of AC1 Glucanase product in two independent studies has demonstrated that AC1 Glucanase product is homogeneously distributed in typical corn/soybean meal based feeds (Ayres *et al.*, 2018 and Jacek *et al.*, 2018).

6.5.4 Stability of the AC1 Glucanase During Pelleting

The stability of the glucanase activity of the AC1 Glucanase product in feed mixtures during pelleting was investigated and reported by Ayres *et al.* (2018). The AC1 Glucanase product was mixed into a batch of feed to prepare diets with a target dose of 500 U/kg. This feed was pelleted using a conditioning temperature of either 80, 85, or 90 C for 10 s and extruded through a 4 x 38 mm pellet die at approximately 0.91 metric ton/h using a 40-horsepower (b) (4) The steam used in the mill had an incoming pressure of 262 kPa. Pellets were cooled using a horizontal belt cooler and ambient air. Pellet conveyance post extrusion and cooling was accomplished with a series of flat bottom drag-chain conveyers and a bucket elevator. Ten 500g samples of the mash feed prior to pelleting and three 500g samples of post-pelleting feeds from each temperature treatment were collected for β-glucanase activity analysis. The β-glucanase activities after pelleting at the different temperatures are presented in Table 25. The results demonstrate that AC1 Glucanase retains nearly 100% activity at 80°C and 85°C pelleting condition, and retains 90% activity after pelleting at 90°C.

Table 25. Glucanase activity in feed samples before (mash) and after (pelleted) pelleting at different temperatures (from Ayres *et al.*, 2018).

Treatment	Activity (unit/kg)		Recovery (%)
	Mean	SD	
Mash	500.45	50.68	100.00%
80°C	551.56	45.31	110.21%
85°C	496.68	38.99	99.25%
90°C	451.58	30.70	90.23%

Another AC1 Glucanase pelleting stability study was conducted and has been reported by Jacek *et al.*, (2018). About 0.4 kg and 1.2 kg of AC1 Glucanase product was mixed into 1500 lb batches of feed to achieve target doses of 100 U/kg and 300 U/kg. Approximately 500 lb of feed was pelleted at either 80, 85, or 90°C in a (b) (4), Master model, with pelletizer flow rate of 1 ton/hr. Feed conditioning was for 15 seconds with 20 psi of steam pressure. Pellets with a diameter of 11/64 mm were extruded and their temperature was measured upon exit from the conditioner. The pellets were cooled and dried prior to bagging. The conditioning temperature and hot pellet temperatures were recorded. Ten 500g samples of post-pelleting feeds were collected for glucanase activity analysis. The glucanase activities in the feed with a target dose of 100 U glucanse/kg after pelleting at the different temperatures are presented as a percentage of the original activity in the respective mash diets in Table 26.

The results of the pelleting stability of AC1 Glucanase in the feed with a target dose of 100 U/kg were reported by Jacek *et al.* (2018) and those from the 300 U/kg feed were not published. The results from both feed groups demonstrated that AC1 Glucanase retains 100% of its activity when pelleted at 80°C for both target doses and retains 83% (100 U/kg) and 92% (300 U/kg) when pelleted at 85°C. When pelleted at 90°C, 82% of the activity was retained for target dose of 100 U/kg, and 79% activity retained for the target dose of 300 U/kg (Table 26).

In summary, the results of three independent pelleting stability studies with corn/soybean meal based feed treated with AC1 Glucanase have demonstrated the thermostability of AC1 Glucanase in high temperature pelleting processes. These studies demonstrated that AC1 Glucanase treated feed retains at least 80% activity after pelleting at 90°C.

Table 26. Glucanase activity in feeds mixed with AC1 Glucanase to a target dose of 100 and 300 U/kg before (mash) and after pelleting at different temperatures. The glucanase activity measured in the mash feed prior to pelleting was used to calculate the percent activity recovered from the feed after pelleting.

Target Dose (U/kg)	Pelleting Temp (°C)	No. of Samples	Avg Activity (U/kg)	Std Dev	Recovery (%)
100	Mash	10	72.21	18.80	100
	80°C	5	80.01	13.57	110.8
	85°C	5	60.17	8.85	83.3
	90°C	5	59.00	11.54	81.7
300	Mash	10	310.05	26.03	100
	80°C	5	314.57	28.11	101
	85°C	5	285.06	35.38	92
	90°C	5	244.37	35.09	79

6.6 GralNzyme® AC1 Glucanase Product Label

GralNzyme® AC1 Glucanase

Description: This product consists of corn meal produced from a genetically engineered variety of corn that produces AC1 Glucanase in the grain. AC1 Glucanase is a thermostable enzyme that improves the digestibility of feedstuffs through reducing the viscosity of the feed in the animal's digestive tract.

Lot Number:

Guaranteed Analysis: This product contains a minimum of β-glucanase activity of 150 units/gram grain. One unit is determined on β-glucan substrate at 80°C and pH 6.5.

Ingredients: Corn meal containing GralNzyme® AC1 Glucanase.

Directions for use in poultry: Add sufficient amount of GralNzymie® AC1 Glucanase per ton of complete feed to deliver 200 to 500 U/kg of feed; if pelleting feed do not exceed 90°C. Store dry at room temperature.

Expiration date: use within 9 months of date of manufacture

Produced by: Agrivida, Inc., 78E Olympia Ave., Woburn, MA 01801, USA

Net weight: 50 lbs

See Material Safety Data Sheet for further information

6.7 Manufacturing Process

The GraINzyme® AC1 Glucanase product is produced by maize genetically engineered to contain a copy of the AC1 Glucanase gene under the regulation of monocot-derived, seed-specific promoters. This results in the production of AC1 Glucanase protein in the grain of maize with little or no production in the leaves, stalks, or other tissues. Therefore, the method of production of the commercial AC1 Glucanase product employs the same agronomic practices as is typically used for the production of conventional maize grain. These include planting maize seed containing the AC1 Glucanase gene into soil once the soil temperature has reached the appropriate temperature for the germination of maize seed, management of the crop using common agricultural practices for the cultivation of maize that may include the application of chemical fertilizers and crop protection chemicals such as herbicides and insecticides that are approved for use on maize, and harvesting by mechanical maize harvesters with a sheller to produce whole maize grain. Alternatively, AC1 Glucanase-producing maize can be grown in a greenhouse with controlled temperature using common practices for the cultivation of maize in a greenhouse or other controlled environments. It is well recognized that using these practices it is possible to produce maize grain in a greenhouse that is nutritionally equivalent to that produced in a field environment.

The whole grain containing AC1 Glucanase is dried to a moisture content of less than 15% and is stored in dry, secure grain storage bins prior to being milled to a course maize meal (~ 2 – 3 mm diameter). Once the AC1 Glucanase grain is milled it is packaged into a secure, labeled container that may include for example a double paper bag with sewn seams containing approximately 20 kg of product or a large heavy plastic tote containing 1 ton of product. The amount of AC1 Glucanase produced in the grain is in the range of 150 to 300 units of glucanase activity (U)/ g. It is expected that 0.7 to 3.3 kg of the AC1 Glucanase product is sufficient to treat one ton of animal feed in order to deliver an effective dose of AC1 Glucanase to improve feed digestibility.

Since the AC1 Glucanase product consists of milled maize grain containing the AC1 Glucanase protein, its nutrient composition is the same as that of typical maize grain. The addition of relatively small quantities of AC1 Glucanase product to typical corn/soy-based diets will replace an equally small amount of the maize that is normally a component of the diet and this substitution will not alter the nutrient composition of the feeds.

6.8 Data inconsistent with a conclusion of GRAS

In this GRAS notification, Agrivida, Inc. has presented all information in its possession and of which it is aware that is relevant and pertinent to its conclusion that the use of the GralNzyme® AC1 Glucanase in poultry is GRAS. Agrivida, Inc. has no information nor is it aware of any information that is inconsistent with, or contradicts, this conclusion of GRAS status for the use of GralNzyme® AC1 Glucanase in the feed of poultry.

6.9 Summary Conclusions

The data and information described in §6.0 above that is the basis for the conclusion by Agrivida, Inc. that the GralNzyme® AC1 Glucanase product is GRAS for use in poultry includes the following:

1. The GralNzyme® AC1 Glucanase is an enzyme and enzymes generally are known to be non-toxic. In all cases of proteins that are toxic, toxicity is derived from the biological mode of action of the protein. The biological mode of action of glucanases is generally recognized to be safe.

2. The history of safe use of glucanases as animal feed additives and in human nutritional supplements is well established and generally recognized. In addition, glucanase enzymes are present in many plant based foods that have been consumed safely by humans for millennia.

3. The safety of the AC1 Glucanase was thoroughly assessed in an Early Food Safety Evaluation conducted by Agrivida, Inc. for this product and this evaluation was reviewed by the U.S. FDA Center for Food Safety and Applied Nutrition. (FDA/CFSAN, 2018).

4. The safety of GralNzyme® AC1 Glucanase is supported by results of a tolerance study described in §6.2.13 in which chickens were fed feed supplemented with 5,000 U glucanase/kg feed without any indications of toxicity (Broomhead *et al.*, 2019).

5. The application of a well used decision tree for evaluating the safety of enzymes used in food and feed (Pariza and Johnson, 2001) to the GralNzyme® AC1 Glucanase product indicates that this product is unlikely to be toxic.

6. The results of poultry feeding trials in which the GralNzyme® AC1 Glucanase was included in the feed at a range of doses and *in vitro* dietary viscosity assays support a conclusion that the GralNzyme® AC1 Glucanase product is safe and functional when included in poultry feed at the doses described (Ayres *et al*, 2018; Jacek *et al.*, 2018; Ayres *et al*, 2019).

Based on the publicly and generally available information described above, Agrivida, Inc. believes that experts in the fields of animal nutrition, toxicology or related fields would agree with the conclusion of Agrivida, Inc. that the GraINzyme® AC1 Glucanase is safe and effective when included in the feed of poultry in the dose ranges described herein.

7.0 References

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8.0 Appendices

8.1 List of Appendices

8.2 Appendix 1

The Annotated Nucleotide Sequence of the T-DNA Locus of Event FG259

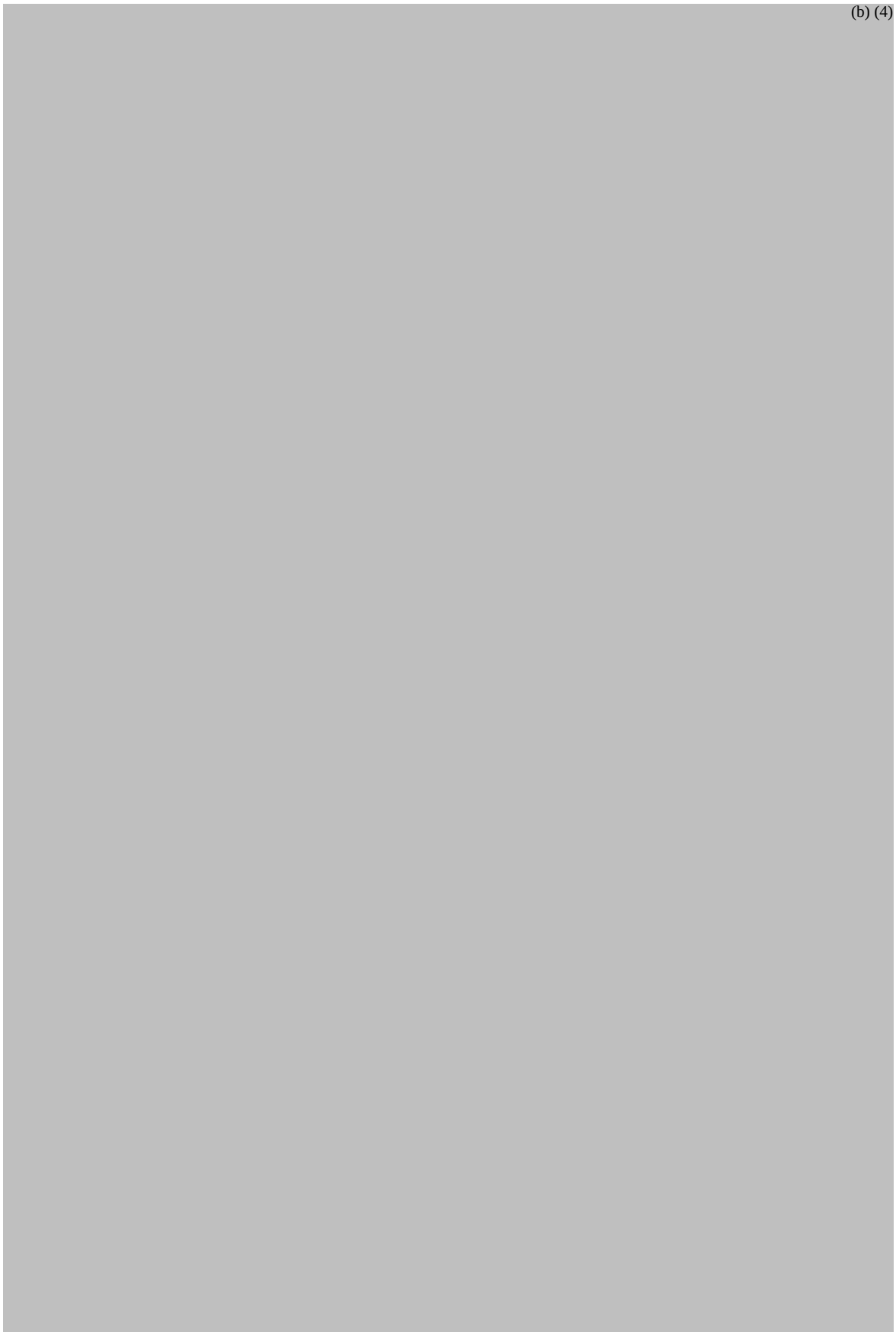
Table 1A. Annotation of the (b) (4). Abbreviations are as listed in Table 1 (Sec 6.1.4)

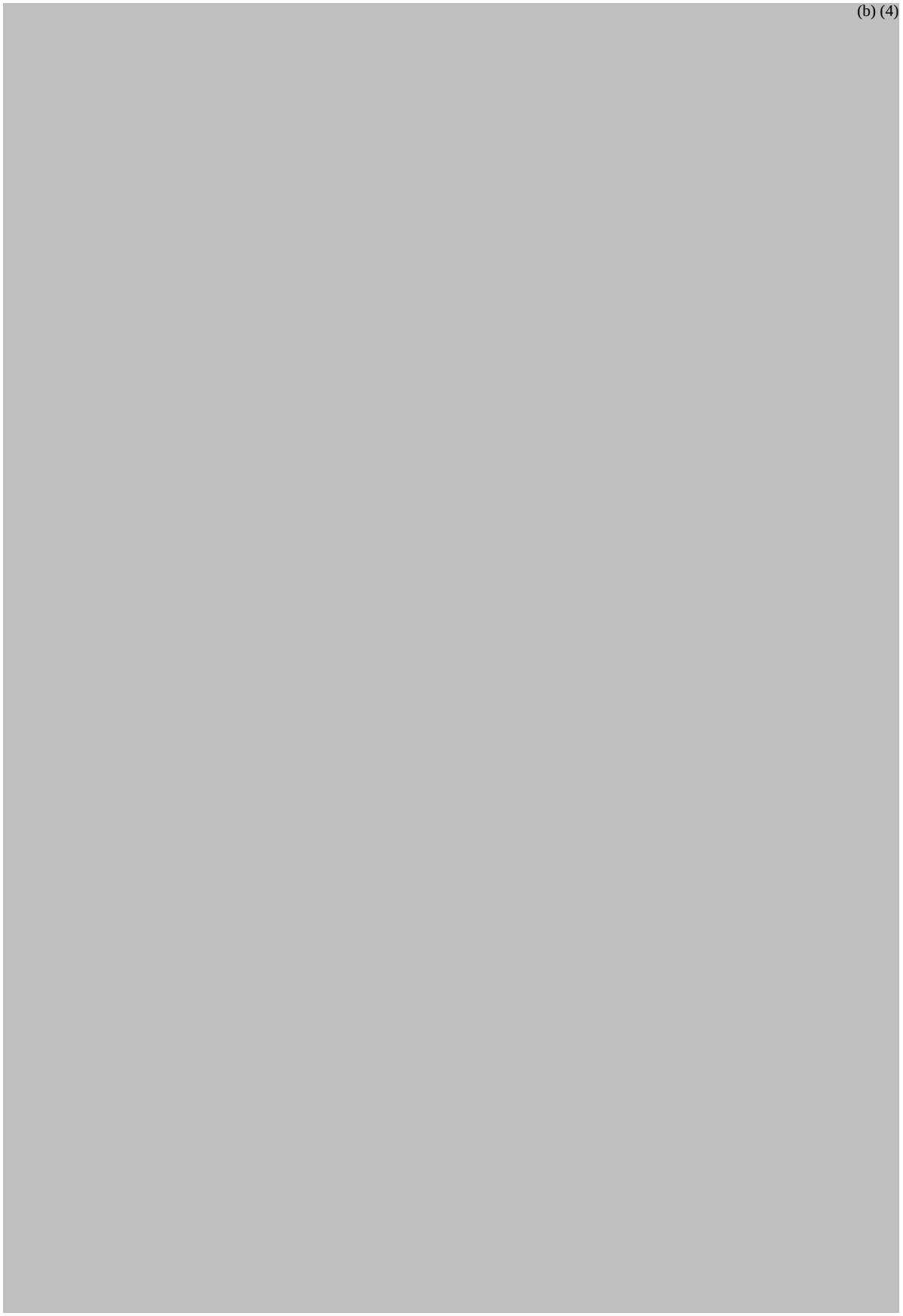
Nucleotide Position		
(b) (4)		

Figure 1A. Nucleotide sequence of the T-DNA insertion locus including maize genomic flanking DNA in Event FG259. Maize genomic DNA sequence is presented in lower case letters while the sequence of the T-DNA insert is presented in upper case letters.

(b) (4)

(b) (4)





8.3 Appendix 2

Characterization of AC1 Glucanase product batches

STUDY TITLE:

CHARACTERIZATION OF AC1 GLUCANASE TEST SUBSTANCES AV_AC1_0070, AV_AC1_0075, AND AV_AC1_0077

AUTHOR;

XUEMEI LI, PHD

STUDY COMPLETED ON JULY 14,2018

PERFORMING LABORATORY:

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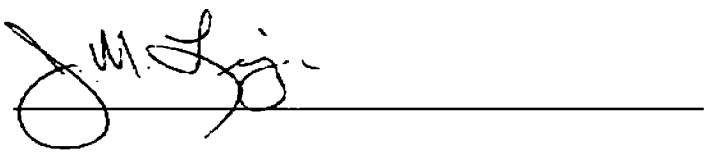
STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

Company: Agrivida, Inc.

Company Agent: James Ligon Date: November 27, 2018

Title: Vice President, Regulatory Affairs and Stewardship

Signature: 

These data are the property of Agrivida, Inc. and, as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute in any other country.

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

The study described in this volume was conducted according to the principles of applicable Good Laboratory Practices as described in 40 CFR 160.

STUDY DIRECTOR:

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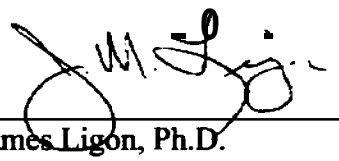

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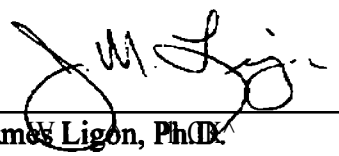

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CHARACTERIZATION OF AC1 GLUCANASE TEST SUBSTANCES AV_AC1_0070, AV_AC1_0075, AND AV_AC1_0077

TEST SUBSTANCE CHARACTERIZATION REPORT AND CERTIFICATE OF ANALYSIS

Test Substance: Ground grain from maize producing the GraINzyme® AC1 enzyme. Test substances are derived from three separate and representative AC1 product batches.

Sample Lot Nos.: AV_AC1_0070
AV_AC1_0075
AV_AC1_0077

SUMMARY

AC1 is produced in the grain of maize through the application of recombinant DNA technologies. Three separate and representative AC1 product batches (designated AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077) were produced using standard agronomic practices for the production of maize. The grain was dried and ground to a coarse meal. The enzymatic activity and characteristics of the AC1 Glucanase produced in the three representative batches were determined. The AC1 Glucanase protein was demonstrated to be a prominent protein in the total protein of all three product batches. Western blot analysis of all three samples revealed a single band of immunoreactive material of the predicted molecular weight of approximately 37.7 kDa.

INTRODUCTION

The purpose of this study was to characterize test substances, AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077, containing the AC1 produced in the grain of maize. Carbohydrases such as AC1 are enzymes that catalyze the depolymerization of nonstarch polysaccharides and, when incorporated into animal feed, increasing the digestibility of nonstarch polysaccharides and reducing the viscosity of digesta. The test substances were prepared from the grain of recombinant maize and are intended for use in animal safety and functionality studies with the AC1 product. Various biochemical parameters

were evaluated to confirm the identity of AC1 in the test substance, as well as its activity and integrity.

MATERIALS AND METHODS

Production of test substances. Three separate representative AC1 product batches were produced from AC1-expressing maize. The product batch numbers, location of planting and dates of planting and harvest are shown in Table 1. Planting the seed and harvest of the grain were performed using commonly used agronomic practices for maize. Cultivation of the AC1-producing maize utilized commonly used agronomic practices for maize including the use of fertilizers, herbicides and pesticides approved for use on maize. After harvest, the grain was dried on the cob for three days until the grain moisture was below 15% at which time it was shelled and placed in labeled containers. The grain was shipped to Agrivida, Inc. (Medford, MA) and stored in separate storage bins prior to being milled in a knife mill (Retch SM100) and sieved through a steel mesh sieve in the mill to produce grain particles less than 1 mm in diameter.

Table 1. Planting locations and dates for the production of three representative AC1 Glucanase product batches.

Product Batch No.	AV_AC1_0070	AV_AC1_0075	AV_AC1_0077
Planting Location	(b) (6)		
Planting Date	11/28/2016	1/20/2016	11/28/2016
Harvest Date	4/27/2017	5/16/2016	4/27/2017

Preparation of extracts. Test substance from each of the three product batches was ground to flour in the knife mill. Protein extracts were prepared as follows. Incubate AC1 extraction buffer (100 mM sodium phosphate, 0.01% Tween 20, pH 6.5) in a water bath at 60°C until the buffer reaches a temperature of 60°C and mix 100 mL warm buffer with 20 g of flour from each batch in a 250 mL plastic flask. Duplicate samples from each product batch were prepared for extraction. Samples were shaken at 60°C for 1 hour. About 1.2 mL sample suspension was transferred into a 1.5 mL tube and centrifuged at 16,000 g for 10 minutes. The supernatant was reserved for analyses.

Enzymatic activity. The AC1 enzyme activity in each of the product batches was assayed according to an Agrivida, Inc. SOP that is described in Appendix 3. Since β-glucanase activity is the primary activity of AC1 protein, a β-glucanase assay was used to determine the AC1 enzyme activity. The glucanase colorimetric assay described in detail in Appendix 3 uses a commercial substrate, azurine-cross linked barley β-glucan (AZCL-Beta-Glucan), from (b) (4) (b) (4) Hydrolysis of this substrate by AC1 enzyme produces water-soluble dye-labeled fragments whose release can be

directly correlated to enzyme activity by measurement of absorbance at 590 nm (A590). The protein extracts produced from the AC1 grain flour samples as described above were diluted 60- and 80-fold and 50 µl of each dilution was mixed with 450 µl of AC1 extraction buffer containing the AZCL-Beta-Glucan substrate. The mixtures were incubated in a water bath at 80°C for 1 hour and mixed with 1 mL of 2% Tris base to terminate the reaction. The reaction was centrifuged at 3,000 g for 10 minutes, and 100 µl of supernatant was removed to a microplate to measure absorbance at 590nm. AC1 enzyme activity (A590/mg) was determined for each batch by dividing the A590 by the amount of flour included in the protein extract (mg). The enzyme activity (A590/mg) can also be converted to the activity units (unit/gram) by multiplying by 9 (Appendix 3 GraINzyme Glucanase AC1 Enzyme Colorimetric Assay Validation).

Specific activity. The specific activity (unit/mg AC1 protein) of AC1 in the test substance material from each product batch was determined. AC1 protein in the aqueous extracts was quantified by ELISA following the protocol from " (b) (4) [redacted] . An aqueous extract of each product batch was diluted 60,000- and 80,000-fold. A series of dilutions of AC1 protein that had been purified from AC1- expressing corn grains were used as protein calibrators to calculate the content of AC1 in each product batch.

Molecular weight determination. SDS-PAGE of the sample extracts was performed as follows. Each extract was diluted 4-fold by mixing 10µl of extract with 30µl of AC1 extraction buffer. A positive control consisting of AC1 purified from AC1- expressing grain was diluted to 50 µg/mL. 15µl of each dilution was mixed with 5µl of Novex NuPAGE 4x LDS sample loading buffer and heated for 10 minutes at 85°C. The heat-treated samples were centrifuged briefly and loaded onto a Novex NuPAGE 4-12% Bis-Tris gel and run in NuPAGE MOPS buffer for about 90 minutes at 100V. In order to visualize the protein bands in the extracts, the gel was placed into 100 mL of 0.1% Coomassie Blue in 10% acetic acid/10% methanol, heated in a microwave oven for 30 seconds, and then shaken for 20 minutes. The gel was rinsed with water and then destained with 10% acetic acid/10% methanol.

Immunoreactivity. To assess the integrity of the AC1 protein in the three AC1 product batches, Western blot analysis was performed. Each extract was diluted 15-fold by mixing 10µl of extract with 140µl of AC1 extraction buffer. A positive control consisting of purified AC1 protein from AC1- expressing grain was diluted to 20 µg/mL. 15µl of each dilution was mixed with 5µl of Novex NuPAGE 4x LDS sample loading buffer and heated for 10 minutes at 85°C. The heat treated samples were centrifuged briefly and the supernatants were loaded onto a gel and electrophoresed as described above. The gel was rinsed in 10 mM CAPS/ 10% methanol transfer buffer for 10 minutes and then electrophoretically transferred to a PVDF membrane for 1 hour at 15 V. The membrane was blocked for 1 hour at room temperature with 5% nonfat milk in TBST (tris-buffered saline with Tween 20). The membrane was then shaken in primary antibody (mouse monoclonal antibody raised against AC1 Glucanase by (b) (4) [redacted] .; 25 mL of a 1:2000 dilution in TBST/5% nonfat milk) overnight at 4°C, followed by three 15 minute washes in TBST. The secondary antibody (25 mL of goat anti-

mouse/HRP, [redacted]; 1:5000 dilution in TBST) was applied with shaking for one hour, followed by three 15 minute washes in TBST. The blot was developed with [redacted] substrate [redacted] The blot image was captured on [redacted] with [redacted] image acquisition software.

RESULTS

Enzymatic activity. The AC1 Glucanase enzyme activity of each of the test substance materials and of corn flour from a non-transgenic control hybrid was determined (Table 2). There was no detectable AC1 activity in the nontransgenic control.

Table 2. AC1 Glucanase activity of three representative AC1 product batches compared to that of grain from nontransgenic control (WT). The enzyme activity is presented as β-glucanase activity units/gram flour.

	Units/g flour	
Product Batch	Mean	SD
AV_AC1_0070	200.20	1.63
AV_AC1_0075	206.75	2.57
AV_AC1_0077	198.11	15.82
WT	0.08	0.09

Specific activity. The quantity of AC1 protein in each of the test substances was determined by ELISA method. The specific activities of each AC1 test substance expressed in units of activity/mg protein are shown in Table 3.

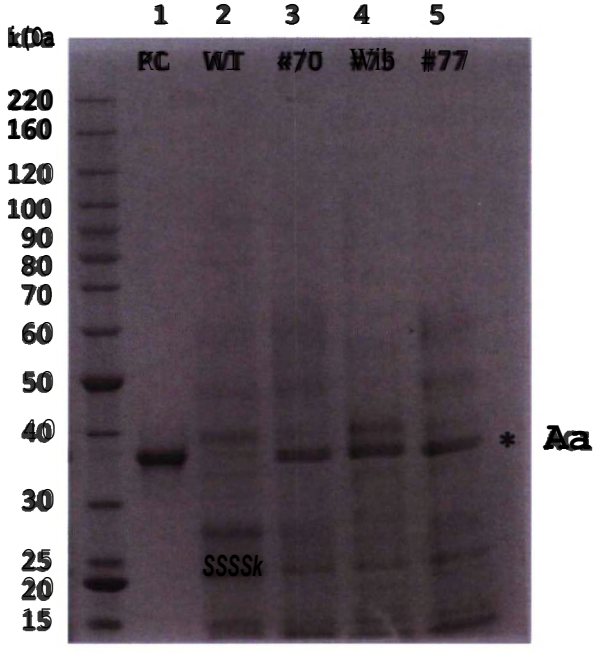
Table 3. AC1 specific activity of three representative AC1 product batches. The enzyme activity was converted to unit/mg of AC1 protein

	Units/mg of AC1 protein	
Product Batch	Mean	SD
AV_AC1_0070	292.61	0.38
AV_AC1_0075	384.17	25.60
AV_AC1_0077	301.38	8.05

Determination of the molecular weight of the AC1 Glucanase protein. An SDS-PAGE gel containing protein extracts from each of the three test substances, protein extracts of corn flour derived from non-transgenic maize, and AC1 purified from AC1-expressing maize grain were stained with Coomassie Blue to enable visualization of the

proteins. Examination of the gel and comparison of the samples demonstrated that there is a prominent protein band in the extracts from all three test substances that is absent in the extract from the conventional corn flour, and that has the same molecular weight as the AC1 purified from AC1-producing maize grain (Figure 1). Comparison of the position of these protein bands in the gel relative to the molecular weight markers included on the gel shows that the prominent protein band in the extracts of the test substances and the purified AC1 protein are approximately 37 kilodaltons (kDa) in size. This estimation of the size of the protein bands compares well with the predicted molecular weight of 37.7 kDa (http://web.expasy.org/compute_pi) for the mature AC1 protein bearing the SEKDEL endoplasmic reticulum retention signal from maize.

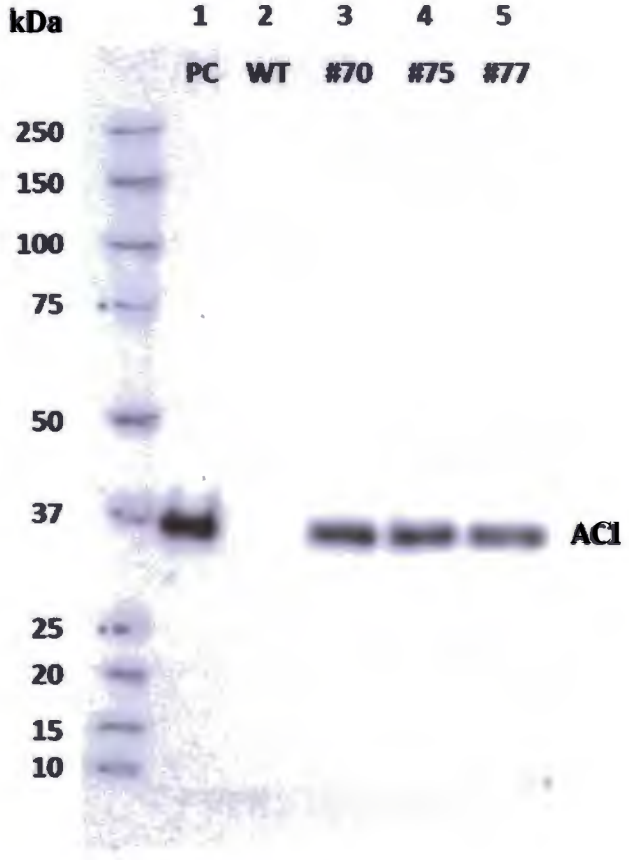
Figure 1. CoomassieBlue-stained SDS-PAGE analysis of AC1.
Lane 1: Positive control (PC) composed of AC1 purified from AC1- expressing maize grain. Lane 2: Negative control composed of protein extracted from grain of non-transgenic maize (WT). Lanes 3,4,5: Protein extracts from three AC1 product batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77, respectively). Molecular weight markers [redacted] (b) (4) were run in the left lane and their sizes in kDa are indicated on the left side of the gel. An asterisk indicates the position of AC1 protein present in the AC1 product batches and positive control (PC).



Immunoreactivity. Western blot analysis of the proteins in extracts from the three test substances was performed using mouse monoclonal anti-AC1 ([redacted] (b) (4).). The results revealed the presence of one immunoreactive protein corresponding to the predicted molecular weight of the AC1 protein (*ca.* 37.7 kDa; Fig. 2). Similarly, the antibody also reacted with the purified AC1 protein control. These results confirm the integrity and identity of AC1 as the prominent protein species present in each of the three

test substances but absent in non-transgenic maize, and confirm its expected molecular weight of approximately 37.7 kDa.

Figure 2. Western blot of protein extracts that were probed with an AC1- specific antibody.
Protein size markers (b) (4) were run in the left lane and their sizes in kDa are indicated on the left side of the gel. Lane 1: Positive control (PC) composed of AC1 purified from AC1- expressing maize grain. Lane 2: Negative control composed of protein extracted from grain of non-transgenic maize (WT). Lanes 3,4,5: Protein extracts from three AC1 product batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77, respectively). Molecular weight markers (b) (4) (b) (4) were run in the left lane and their sizes in kDa are indicated on the left side of the gel. AC1 protein present in the AC1 product batches and in the positive control (PC) is indicated.



RECORDS RETENTION: Raw data, the original copy of this report, and other relevant records are archived at Agrivida, Inc., 200 Boston Avenue, Medford, MA, USA 02155.

STUDY PERSONNEL: Analytical work reported herein was conducted by Xuemei Li, Ph.D., Agrivida, Inc., 200 Boston Avenue, Medford, MA, USA 02155.

REFERENCES

1. Federal Register, Part IV, 40 CFR, Part 160, 17 August 1989 and subsequent revisions.
2. Megazyme (2012). Assay of endo-β-glucanases using beta-glucazyme tablets. Megazyme International Ltd, Wicklow, Ireland. Available at: https://secure.megazyme.com/files/Booklet/B-BGZ_DATA.pdf

8.4 Appendix 3

ACI Glucanase Activity Colorimetric Assay and Validation

Glucanase Colorimetric Assay

The procedure for measuring the activity of glucanase enzymes, including β-glucanase using an azo-barley-glucan as a substrate has been described by McCleary and Shameer (1987). This *in vitro* assay produces a blue-colored solution by hydrolyzing the substrate, changing the color of the reaction supernatant, which can be recorded as a change in absorbance at 590 nm. The rate of the color change is correlated directly to enzyme activity.

This *in vitro* colorimetric glucanase enzyme activity assay has proven to be a simple and reliable method. The enzyme activity data obtained from this method correlated well with the *in-vivo* response to enzyme supplementation of barley-based diets when fed to young chicks (Rotter et al., 1990).

The glucanase colorimetric assay described herein uses a similar commercial substrate, azurine-cross linked barley β-glucan (AZCL-Beta-Glucan) from Megazyme (Wicklow, Ireland). Hydrolysis of this substrate by Agrivida GraINzyme® ACI Glucanase enzyme produces water soluble dyed fragments, and the rate of release of these dyed fragments, which increases in absorbance at 590 nm, is correlated directly to enzyme activity (Megazyme, 2012).

References

McCleary B.V. and I. Shameer (1987). Assay of malt β-Glucanase using azo-barley glucan: An improved precipitant. J. I. Brewing 93:87-90.

Rotter, B.A., R.R Marquardt, W. Guenter and G.H. Crow (1990). Evaluation of three enzymic methods as predictors of in-vivo response to enzyme supplementation of barley-based diets when fed to young chicks.. J. Sci. Food Agric. 50:19-27.

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https://secure.megazyme.com/files/Booklet/TBGZ_DATA.pdf

1. Limit of Detection (LOD)

The limit of detection for the AZCL-barely-beta glucan assay was determined by calculating the mean A590 and standard deviation of the mean for multiple negative samples, each of which was derived from corn that does not produce AC1 Glucanase enzyme ("WT corn"). The LOD for the assay can be defined as the mean A590 of these negative samples plus three standard deviations.

Table 1. Data from assays of WT corn samples and calculations for LOD (mean A590 plus 3 standard deviations)

Negative Seed A590 Readings

0.144	0.097	0.115	0.094	0.091	0.093	0.099	0.097	0.093	0.087	0.086	0.088
0.095	0.101	0.111	0.087	0.095	0.086	0.091	0.086	0.088	0.084	0.085	0.089
0.085	0.084	0.085	0.09	0.083	0.085	0.097	0.087	0.089	0.09	0.08	0.077
0.087	0.099	0.084	0.087	0.087	0.087	0.105	0.103	0.094	0.092	0.084	0.078
0.078	0.085	0.081	0.116	0.097	0.09	0.108	0.092	0.094	0.088	0.083	0.104
0.086	0.09	0.083	0.087	0.096	0.095	0.09	0.086	0.098	0.084	0.076	0.094
0.099	0.163	0.125	0.092	0.08	0.079	0.084	0.077				

n =	Mean OD	Std dev	3 std dev	LOD
80	0.092263	0.013513	0.040539	0.132801

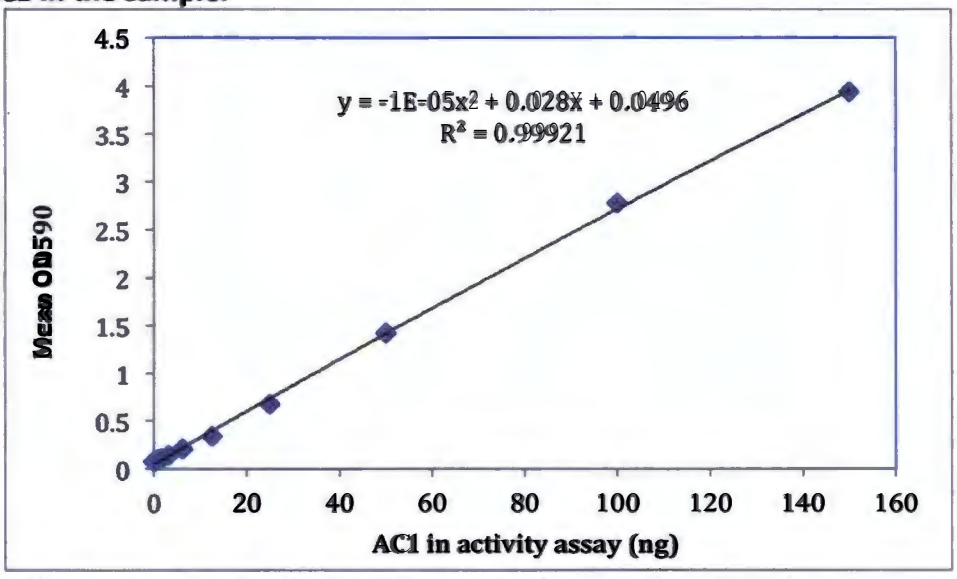
By comparing the A590 of the LOD to a standard curve of AC1 Glucanase calibrators, it is possible to express the LOD in terms of nanograms of AC1 Glucanase protein. A 12 series dilutions of AC1 Glucanase were used in replicate assays involving AZCL-barley-beta-glucan (as described in Protocol I), and the A590 values from each sample were recorded (Table 2). Applying a quadratic best-fit curve to the data created a standard curve that can be used to infer the correlation between A590 and ng of AC1 Glucanase protein (Figure 1).

Table 2. A590 data for calculating the standard curve.

Activity of the calibrators (A590)													
AC1 in activity assay (ng)	0	0.3	0.5	1.0	1.6	3.1	6.3	12.5	25.0	50.0	100.0	150.0	200.0
Rep_1	0.077	0.08	0.088	0.098	0.11	0.142	0.212	0.348	0.709	1.48	2.825	3.961	Plateaued
Rep_2	0.077	0.076	0.084	0.094	0.105	0.133	0.195	0.331	0.641	1.364	2.725	3.91	Plateaued
Average	-3E-08	0.078	0.086	0.096	0.108	0.138	0.204	0.340	0.675	1.422	2.775	3.936	

*Plateaued: these values were above the maximum detection threshold of the spectrophotometer.

Figure 1. Standard curve of AC1 Glucanase activity vs the amount of AC1 Glucanase in the assay. The quadratic equation is used to calculate the amount of AC1 in the sample.



Using the quadratic equation for the best-fit standard curve, the LOD value of 0.132801 A590 can be converted to 2.97 ng of AC1 protein.
From these results, we infer that the LOD for the GralNzyme® Glucanase AC1 activity detection is about 3 ng.

2. Assay Sensitivity and Matrix Effects

To determine whether the beta-glucanase activity colorimetric assay is affected by the matrix when AC1-expressing corn product is mixed with animal feed, AC1 Glucanase protein was assayed in the presence of either WT (non-AC1) corn flour, or the formulated animal feed.

2.1 Mixing and Recovery of AC1 Glucanase activity from WT corn product

Extraction buffer was spiked with AC1 Glucanase protein at concentrations of 5, 10, 25, 50, 70, 125, 250, 500, 1000, 2000 and 3000ppb. 2.5ml of each spiked extraction buffer was mixed with 0.5g of ground non-AC1 Glucanase corn in triplicate. The remaining spiked buffer and the buffer/corn mixes were placed on a temperature-controlled shaker, shaking for 1 hour at 250rpm and 80°C. After 1 hour shaking, the buffers and the buffer/corn mixes were removed from the shaker, and the buffer/corn mixes were subjected to centrifugation. 50 µl of each buffer in triplicate or each supernatant (protein extract) from buffer/corn mixes was used for glucanase colorimetric assay. The amount of AC1 Glucanase protein used in enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 and 150 ng. The sample extraction and activity assay was carried out according to the standard protocol in Protocol I. AC1 Glucanase enzyme recovery is

the average of triplicate assay activities from the product matrix relative to that from AC1 Glucanase spiked buffer.

Table 3. Data from assay of AC1 Glucanase in the presence of ground WT corn.

AC1 spiked in extraction buffer	AC1 in activity assay	Extraction Buffer (A590)			Product Matrix (A590)			% Average Recovery
ppb	ng	Replicate_1	Replicate_2	Replicate_3	Replicate_1	Replicate_2	Replicate_3	(Matrix/Buffer)
0	0	0	0.002	0	0	0	0	0.00%
2.5	0.25	0	0.003	0.001	0	0	0	0.00%
5	0.5	0.003	0.003	0.004	0.009	0.01	0.011	300.00%
12.5	1.25	0.02	0.023	0.017	0.026	0.036	0.021	138.33%
25	2.5	0.029	0.03	0.027	0.045	0.047	0.047	161.63%
35	3.5	0.064	0.067	0.06	0.078	0.082	0.073	122.15%
62.6	6.25	0.102	0.109	0.11	0.115	0.138	0.147	125.09%
125	12.5	0.26	0269	0.267	0.309	0.284	0.29	110.95%
250	25	0.527	0.599	0.575	0.655	0.61	0.591	109.30%
500	50	1.276	1.341	1311	1.406	1.428	1.391	107.58%
1000	100	2525	2.724	2.499	2.793	2.666	2.454	102.27%
1500	150	3.888	Plateaued	Plateaued	Plateaued	Plateaued	3.682	94.70%

*Plateaued: these values were above the maximum detection threshold of the spectrophotometer.

The recoveries ranged between 125% and 95% for samples with AC1 Glucanase protein between 3.5 and 150ng. At 3.5ng, which was close to the LOD, the average recovery was about 122% suggested that very little product matrix effect was present. Samples with 1.25 ng enzyme or lower resulted in recoveries ranging between 138% and 300%, suggesting the assay is less sensitive at such low enzyme inclusion.

2.2 Spiking and Recovery of AC1 activity from feed

To determine whether a complex feed mixture, such as that used in many poultry diets, might interfere with the sensitivity of the AC1 Glucanase assay, serial dilutions of the enzyme were again prepared in extraction buffer and mixed with samples of feed, then assayed for recoverable enzyme activity. For the feed sample, a corn/soybean feed (no AC1 Glucanase addition) was milled to less than 1.0 mm particle size. For the serial dilutions, extraction buffer was spiked with AC1 Glucanase protein at concentrations of 2.5, 5, 10, 25, 50, 70, 125, 250, 500, 1000, and 1500ppb, and 225ml of each buffer was mixed with 0.5g of feed sample in triplicate. The remaining spiked buffer and the buffer/feed mixes were placed on a temperature-controlled shaker, shaking for 1 hour at 250rpm and 80°C. 100ul of supernatant ("extract") was removed from each sample and was used in the standard assay as described in Protocol III. The amount of AC1 Glucanase protein used in enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 and 150ng, respectively. AC1 Glucanase enzyme recovery is the average of triplicate assay activities from the feed matrix relative to that from AC1 Glucanase spiked buffer.

Table 4. Data from assay of AC1 Glucanase in the presence of poultry feed.

AC1 spiked in extraction buffer	AC1 in activity assay	Extraction Buffer (A590)			Feed Matrix (A590)			% Average Recovery (Matrix/Buffer)
ppb	ng	Replicate_1	Replicate_2	Replicate_3	Replicate_1	Replicate_2	Replicate_3	
0	0	0	0	0	0	0	0	0.00%
2.5	0.25	0.001	0.006	0.003	0	0	0	0.00%
5	0.5	0.007	0.01	0.007	0	0	0	0.00%
12.5	1.25	0.02	0.022	0.019	0.006	0	0	9.84%
25	2.5	0.051	0.053	0.05	0.05	0.053	0.051	100.00%
35	3.5	0.116	0.112	0.115	0.117	0.109	0.104	96.21%
62.6	6.26	0.278	0.284	0.264	0.26	0.28	0.268	97.82%
125	12.5	0.287	0.265	0.264	0.334	0.308	0.312	116.91%
250	25	0.521	0.504	0.496	0.552	0.568	0.58	111.77%
500	50	1.313	1.259	1.168	1.408	1.327	1.259	106.79%
1000	100	2.395	2.418	2.401	2.793	2.642	2.59	111.24%
1500	150	Plateaued	Plateaued	3.762	Plateaued	Plateaued	Plateaued	Plateaued

*Plateaued: these values were above the maximum detection threshold of the spectrophotometer.

From these observations, recoveries of AC1 Glucanase from samples that included between 2.5 and 100ng enzyme ranged between 96% and 117% in the presence of ground feed. When the enzyme was included at 2.5ng, the average recovery was 100% suggesting that presence of the feed matrix had very little effect on the sensitivity of the assay. Samples that contained 1.25ng of enzyme or less resulted in poor recovery, suggesting the assay is less sensitive at such low levels of AC1 Glucanase inclusion. Based on the data from experiments in which AC1 Glucanase activity was measured after mixing the enzyme with milled corn or feed, the LOQ of 3.5 ng is confirmed.

2.3 AC1 enzyme activity recovery from the formulated feed diets

To test whether the glucanase colorimetric assay can reliably detect AC1 Glucanase enzyme activity from industrial-type diets formulated with ground AC1 Glucanase-expressing corn meal, the AC1 Glucanase activity in the diets was first compared with that from a standard curve of AC1 Glucanase protein (Table 5 and Figure 2) to determine the amount of AC1 Glucanase protein presented in each diet (Table 7). Then the AC1 Glucanase content in the aqueous extracts from these diets was quantified with a proprietary ELISA plate (Table 6). The amount of AC1 Glucanase protein detected by these two methods was compared to determine the reliability of the glucanase colorimetric assay method (Table 7).

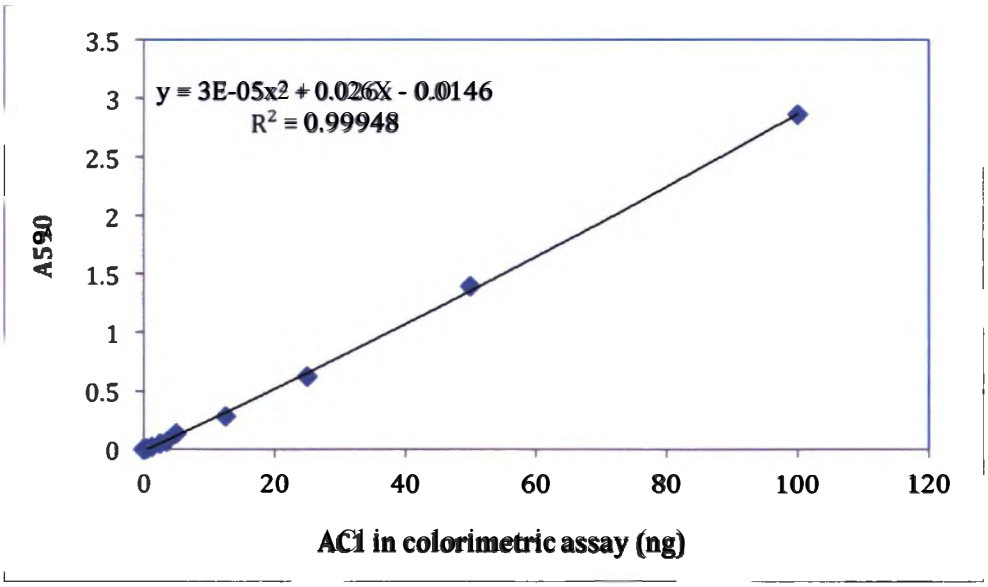
To generate the standard curve, as stated in section 2.2, AC1 Glucanase protein was mixed with extraction buffer at 2.5, 5, 12.5, 25, 35, 62.5, 125, 250, 500, 1000 and 1500ppb, and 2.5ml of each buffer was mixed with 0.5g of the control diet (no AC1 Glucanase addition). The amount of AC1 Glucanase protein used in each enzyme assay

was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 or 150ng in 100ul of protein extract from control diet. The sample extraction and activity assay was carried out according to the standard protocol in Protocol 1.
A standard curve of A590 values reflecting enzyme activity recoveries from the spiked feed vs. the nanograms of AC1 Glucanase used in the enzyme activity assay is shown below (Table 5 and Figure 2).

Table 5. Data from AC1 Glucanase Standard Curve

The amount of AC1 protein in the extracts from the spiked control diet was added to enzyme assay												
(ng)	0	0.25	0.5	1.25	2.5	3.5	5	12.5	25	50	100	150
AS90	0	0	0.004	0.021	0.046	0.065	0.136	0.281	0.624	1.391	2.861	Plateaued

Figure 2. Plot of AC1 Glucanase Standard Curve and Quadratic equation for Best-Fit Curve



The diet samples used for these test were an industry type starter diet formulated with 0, 5, 50, 100, 200 and 400 units corn-expressed AC1 Glucanase /kilogram of diet (see below for the definition of units). After preparing the diets, each was ground by a knife mill, protein was extracted, and AC1 Glucanase enzyme activity was assayed according the standard protocol in Protocol 1.
The amount of AC1 Glucanase protein in the extract was quantified with a proprietary ELISA plate as listed in the Table 6 below. The ELISA assay protocol is presented in Protocol 2.

Table 6. AC1 Glucanase Detection via ELISA

Formulated Diets	Target Dose Unit/kg	ELISA Detected AC1 in protein extract ng/mL	
		Ave	Std
Diet_1	0	1.89	0.34
Diet_2	5	2.61	0.78
Diet_3	50	45.14	7.23
Diet_4	100	103.88	15.66
Diet_5	250	220.50	41.26
Diet_6	500	475.90	158.53

AC1 Glucanase activities measured via the colorimetric (A590) assay in extracts from the formulated diets of feeding trial #43 are shown in Table 7 below. For example, in Diet_3, which was formulated with a target dose of 50 unit AC1 Glucanase/kg, the activity of 0.1±0.006 A590 was detected. This A590 value is converted to 4.39ng based on the standard curve, and this number is very close to the AC1 protein that was detected by ELISA (4.51ng). In Diet_4, which was formulated with a target dose of 100 unit AC1 Glucanase/kg, an A590 value of 0.24±0.033 was obtained which can be converted to 9.68ng from the standard curve, and this number is very close to the AC1 Glucanase protein detected by ELISA study (10.39ng).

Table 7. Detection of AC1 Glucanase in the formulated feed

Formulated Diets	Target Dose Unit/kg	A590 Ave	A590 Std	ng of AC1 in assay Converted from the Std Curve	ng of AC1 in assay Based on ELISA Assay
Diet_1	0	0.002	0.002	0.64	0.19
Diet_2	5	0.002	0.000	0.64	0.26
Diet_3	50	0.100	0.006	4.39	4.51
Diet_4	100	0.240	0.033	9.68	10.39
Diet_5	250	0.644	0.071	24.63	22.05
Diet_6	500	1.308	0.037	48.19	47.59

From these results, it is concluded that the AC1 Glucanase activity can be detected reliably in formulated poultry diets via the colorimetric assay and that the results correlate well with those of an AC1 Glucanase-specific ELISA assay.

Converting colorimetric absorbance values to activity units

One Unit of AC1 Glucanase activity is defined as the amount of enzyme required to release one micromole of reducing sugar equivalents such as glucose from 1% barley-β-glucan, at pH6.5 and 80°C.

The β-glucanase unit (BG unit) activity assay involves multiple steps (as described in Protocol 3) and is less accurate with feed samples, especially when the target dose is lower than 300 unit/kg. This is probably due to the presence of endogenous enzyme activities and reducing sugars in feed, which can interfere with the unit assay. As the colorimetric assay is relatively simple, sensitive and reliable, we use colorimetric absorbance values to infer unit values, and will market the AC1 Glucanase product using the BG unit as a descriptor of activity. To determine the mathematical relationship between values from the colorimetric and unit assays for AC1 Glucanase, we prepared a serial dilution of AC1 Glucanase protein, and measured the activity via both assays. The amount of AC1 Glucanase protein was also measured via ELISA so that both activities could be correlated as a function of amount of AC1 Glucanase protein.

AC1 Glucanase protein dilutions were first measured in the AC1 Glucanase colorimetric assay and then via ELISA (Table 8), and the amount of enzyme activity was calculated per mg AC1 Glucanase protein. Averaging all 10 measurements showed AC1 Glucanase activity was about 50,461 ± 11,347 A590/mg AC1.

Table 8. Enzyme activities of a serial dilution of ACI Glucanase protein from colorimetric assay and the protein concentrations in the diluents measured by ELISA.

ng of ACI in colorimetric assay	ACI Activity (A590)	mg of ACI in the assay based on ELISA data	A590/mg ACI
0.5	0.011	3.06102E-07	35,935.77
1	0.024	6.74523E-07	35,580.68
2	0.049	9.69415E-07	50,545.93
3	0.066	1.42217E-06	46,407.94
4	0.1175	2.21927E-06	52,945.22
5	0.1385	3.15029E-06	43,964.17
10	0.3	6.72132E-06	44,634.09
20	0.668	1.0013E-05	66,713.31
25	0.9355	1.46043E-05	64,056.41
40	1.5875	2.4871E-05	63,829.31

When these same dilutions of ACI Glucanase protein were used in the activity unit assay (Protocol 3), ACI Glucanase activity was calculated as the values of μmol of glucose released per minute per gram of ACI Glucanase, and the average activity is about 456,461 ± 109,990BG unit/g ACI.

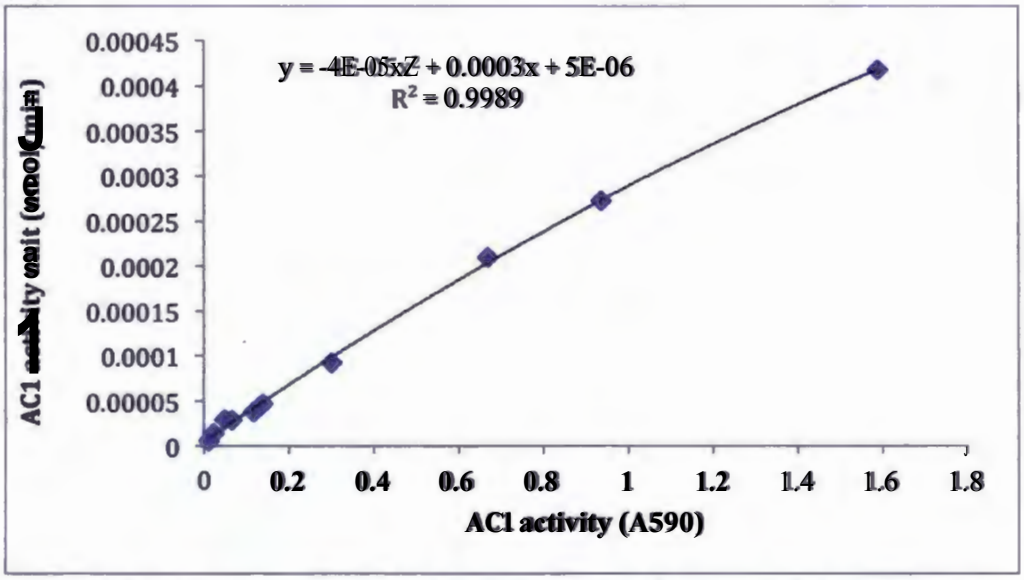
Table 9. Enzyme activities of a series diluted ACI Glucanase protein from BG unit assay and the protein concentrations in the diluents measured by ELISA.

ng of ACI in colorimetric assay	Activity (umol/min)	g of ACI in A590 assay based on ELISA	unit/g ACI
0.5	5.49891E-06	3.06102E-10	431,143.62
1	1.39237E-05	6.74523E-10	495,415.12
2	2.92756E-05	9.69415E-10	724,781.00
3	2.81523E-05	1.42217E-09	475,086.76
4	3.75132E-05	2.21927E-09	405,680.16
5	4.63124E-05	3.15029E-09	352,823.56
10	9.22432E-05	6.72132E-09	329,375.19
20	0.00020888	1.0013E-08	500,661.15
25	0.000271785	1.46043E-08	446,637.97
40	0.000417628	2.4871E-08	403,001.83

Comparing the results from the two ACI Glucanase assays, on average 1 A590/mg ACI Glucanase corresponds to about 9 units/g ACI Glucanase.

Directly comparing the results from the colorimetric and unit assays of purified proteins shows that these two assays are well-correlated, as shown in Figure 3.

Figure 3. Correlation between colorimetric and unit assays of purified AC1 Glucanase protein.



To test whether the relationship between the colorimetric and unit assays was preserved in more complex samples, both assays were used to measure AC1 Glucanase activity in grains with different levels of AC1 Glucanase protein expression. Table 10 illustrates glucanase activity data from 49 different samples of AC1 Glucanase corn products using both the colorimetric assay and the unit assay. The average ratio between unit/g value and A590/mg value is about 9, i.e. 9 unit/g activity value equals to 1 A590/mg activity value.

Table 10: AC1 Glucanase activity in grain samples measured by both colorimetric and unit assays

Sample ID	AC1 activity		Conversion rate
	unit/g	A590/mg	unit/g: A590/mg
1	134.59	21.29	6
2	128.88	21.42	6
3	161.14	21.32	8
4	160.44	23.02	7
5	99.39	9.95	10
6	173.13	17.44	10
7	134.32	10.40	13
8	113.12	9.92	11
9	291.55	24.93	12
10	165.07	14.42	11
11	59.31	7.30	8
12	48.68	6.89	7
13	48.87	8.48	6
14	458.95	52.03	9
15	72.09	10.24	7
16	70.41	12.68	6
17	68.54	12.60	5
18	73.77	11.92	6
19	81.53	10.87	8
20	72.37	12.25	6
21	68.26	11.42	6
22	66.77	9.92	7
23	87.88	11.76	7
24	263.85	25.66	10
25	208.72	19.14	11
26	169.21	15.38	11
27	57.20	3.96	14
28	618.18	64.95	10
29	569.66	66.54	9
30	170.82	18.54	9
31	164.15	16.44	10
32	172.93	16.43	11
33	181.24	17.20	11
34	180.54	16.89	11
35	176.32	16.14	11
36	194.35	18.00	11

37	188.97	16.88	11
38	149.32	19.83	8
39	131.41	17.76	7
40	130.86	18.12	7
41	152.23	21.30	7
42	144.66	20.38	7
43	145.23	20.90	7
44	129.65	17.20	8
45	133.33	17.56	8
46	140.26	17.82	8
47	307.88	41.38	7
48	300.17	42.42	7
49	338.94	43.90	8
Average			9

From these observations, we conclude that the use of the colorimetric assay (A590/mg) to calculate the number of units (BG unit/g) present in the AC1 Glucanase product or in formulated diets is accurate and justified. Table 11 provides an example of converting the colorimetric assay values (A590/g) from formulated poultry diets to activity units (unit/kg). The activity recovery is equal to converted units relative to the target units.

Table 11: Estimates of BG units in formulated diets

Trial # 86	Target	AC1 Glucanase Activity in the Diet		
	unit/kg	A590/g	Converted to unit/kg	% Activity recovery
Diet_1	0	0	0	0.00%
Diet_2	0	0	0	0.00%
Diet_3	50	5.27	47.45	94.90%
Diet_4	100	9.06	81.50	81.50%
Diet_5	200	18.63	167.70	83.85%
Diet_6	400	48.88	439.95	109.99%

The glucanase colorimetric assay can also detect activities from commercial enzyme products

To demonstrate that the glucanase colorimetric assay described herein does not have limited application only on Agrivida AC1 Glucanase, we tested three digestive enzymes (commercially available as human nutrition supplements) using the colorimetric assay protocol. The enzyme content in a capsule of each product was hydrated in 2 ml water, and then was diluted 100-fold before using 50 µl for glucanase colorimetric assay at 37°C and 80°C. β-glucanase activity was detected in these products, and the activity was higher at 80°C assay temperature than that at 37°C (Table 12).

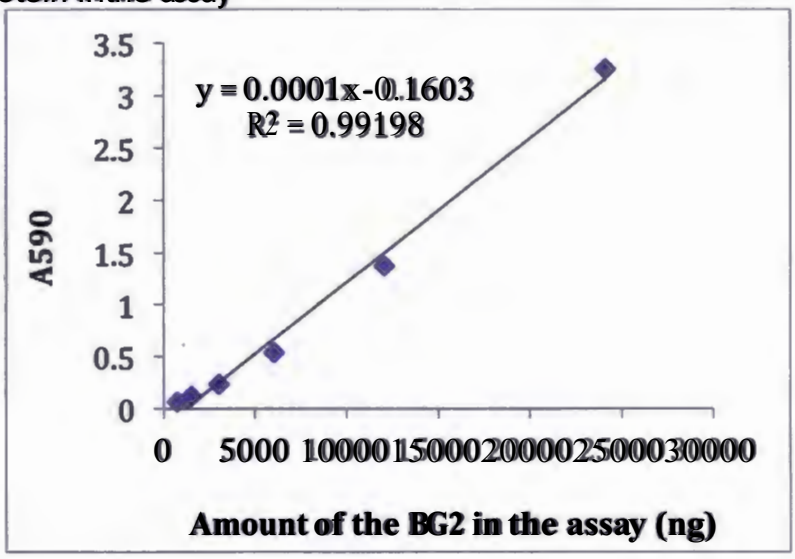
Table 12. Glucanase colorimetric assay detected activities in commercial enzyme products

Product Brand	Super Digestive Enzymes	Enzymatrix	Digestive Enzymes Ultra
Manufacturer	GNC	100naturals	Pure encapsulations
Labeled β-glucanase unit (BGU)	N/A	65	20
A590 (37°C)	0.102	2.321	0.517
A590 (80°C)	0.2665	Plateaued	1.1035

The glucanase colorimetric assay was also applied to a commercial thermostable β-glucanase enzyme (b) (4) A590 absorbance of BG2 correlates well with the amount of enzyme in the assay as shown in Figure 4.

These observations confirm that the colorimetric assay correlates well with the unit assay for β-glucanases, and that A590 can be used to measure enzyme activity in grain, feed, and other samples.

Figure 4. Correlation between colorimetric assay activity (A590) and the amount of BG2 protein in the assay



Protocol 1. GraINzyme® AC1 Glucanase Colorimetric Assay

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model: TP650-9)
1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)
1.1.3. IKA® Tube Mill 100 Control (b) (4)
1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43
1.1.5. Eppendorf benchtop clinical centrifuge Model 5810 or equivalent
1.1.6. Laboratory gloves
1.1.7. Pipette tips (b) (4)
(b) (4)
Calibration date:_____ Expiration Date:_____
1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20µl, 100µl and 200µl)
Calibration Date: _____ Expiration Date:_____
1.1.9. 4.5 ml TallPrep Tubes (b) (4)
1.1.10. 50mL Reagent Reservoirs (b) (4)
1.1.11. 96-well Plate Covers (b) (4)
1.1.12. 96 well block, 2ml (b) (4)
1.1.13. Flat bottom 96 well plate (b) (4)
1.1.14. Water bath
Manufacturer: Benchmark
Model number: B2100-12
Serial number: MBG6120U-148
1.1.15 1L glass bottles
1.1.16 500ml glass bottles
1.1.17 Aluminum foil
1.1.18 Timer
1.1.19 Bench top centrifuges
Manufacturer: Spectrafuge
Model number: C2400-B
Serial number: D812826 and D812827
1.1.20 Permanent markers for labeling
1.1.21 Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492
1.1.22 Metal spatula
1.1.23 pH 4.0 Calibration Solution (b) (4)
1.1.24 pH 7.0 Calibration Solution
1.1.25 pH 10.0 Calibration Solution
1.1.26 Balance

Manufacturer: Mettler
Model number: PM4000
Serial number: F80835

1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number: Synergy HT
Serial number: 191338

1.1.28 pH meter
Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 ml conical centrifuge tube (b) (4)

1.1.30 Micro centrifuge tubes, 2.0 ml (b) (4)

1.1.31 PETG Flasks, 250 ml (b) (4)

1.2 Reagents

1.2.1 200mM Phosphate Stock Buffer, pH 6.5 (2L)

1.2.1.1 1M monosodium phosphate, monohydrate (ACROS Organics, AC389870000, MW 119.98g/mol)
Weigh out 119.98 g and dissolve in distilled water to a final volume of 1L.

1.2.1.2 1M disodium phosphate (Sigma, S5136, MW 141.96g/mol).
Weigh out 141.96 g and dissolved in distilled water to a final volume of 1L.

1.2.1.3 Mix 170ml monosodium phosphate, monohydrate with 230ml disodium phosphate, making pH 6.5. Adjust volume to 2L.

1.2.1.4 Filter buffer and store the buffer at 4C.

1.2.2 Extraction Buffer: 100mM NaPi, pH 6.5, 0.01% Tween 20 (1L)

1.2.2.1 Mix 500ml Phosphate stock buffer with 500ml deionized water

1.2.2.2 Add 0.1ml of concentrated Tween-20

1.2.3 2% Tris-base (1L)

1.2.3.1 20g Tris base

1.2.3.2 Adjust volume to 1L with deionized water

1.2.4 Beta-Glucazyme tablets (Megazyme, Cat# T-BGZ1000)

2 Experimental Procedures

2.1 Sample Preparation, Protein Extraction and Extract Dilution

2.1.1 Sample Preparation

2.1.1.1 Grind negative corn seeds for LOD detection in IKA Tube Mill, 130,000 rpm for 1 minute, which produces 94% product with <0.6 mm grind size

2.1.1.2 Grind corn grains and feed in the Knife mill to less than 1.0 mm grind size for protein extraction and activity test

2.1.2 Protein extraction

2.1.2.1 Extract protein by adding 5 volumes Extraction Buffer which has been pre-warmed at 60°C (e.g. 10 ml Extraction Buffer to 2 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers at 60°C, 250 rpm for 1 Hour After one hour extraction, vortex container for 10 seconds before centrifuging the aqueous extraction at 16,000 g for 10 min. The supernatant is saved for the enzyme activity colorimetric assay

2.1.2.2 Purified protein as calibrator; GralNzyme® AC1 Glucanase protein was purified from AC1-expressing corn grains, and was stored in 50mM MES, 150mM sodium chloride, 40% glycerol, pH 6.3 buffer at -20°C.

2.1.3 Protein dilution for activity assay

2.1.3.1 Protein extract from the negative samples is directly used for activity detection without further dilution

2.1.3.2 Protein extract from AC1 Glucanase -expressing corn product is diluted from 60-fold to 240-fold

2.1.3.3 Protein extract from mash or pellet feed is either not diluted or diluted from 2-fold to 24-fold in the Extraction Buffer

2.1.3.4 The purified AC1 protein is diluted to 5, 10, 20, 31.3, 62.5, 125, 250, 500, 1000, 2000, 3001, and 4000 ng/mL or ppb in the Extraction Buffer

2.2 Colorimetric Activity Assay

Pre-heat the water bath to 80°C. For the AC1 Glucanase *product* activity assay, mix 1 tablet of Beta-Glucazyme substrate with 50ul of the protein extract from product or purified protein (calibrator) or buffer blank, and 450ul extraction buffer in a 96 well block. For the AC1 activity assay *in feed*, mix 1 tablet of Beta-Glucazyme substrate with 100ul of the protein extract from feed or buffer blank, and 400ul extraction buffer in a 96 well block. Incubate the block at 80°C for 1 Hour. After the incubation, add 1mL of Tris Base to stop the reaction. Centrifuge the mixture at 4000g for 10min, then remove 100ul supernatant to a flat bottom microplate to record A590 using a microplate reader.

3. Results Calculation

3.1 Sample activity calculation

3.1.1 Average A590 readings of the buffer blanks

3.1.2 Subtract the average blank A590 reading from each sample (A590')

3.1.3 Multiply A590' by dilution factors (A590")

A590" = A590' x Extract dilution x Assay dilution

Where:

Extract dilution = 60 to 240 (product assay); 1 to 24 (feed assay)

Assay dilution for product = Volume of buffer used for sample extraction (mL)/Volume of protein extract used in enzyme reaction (mL), where 0.05 mL protein extract was used in product activity assay
Assay dilution for feed = Volume of buffer used for sample extraction (mL)/Volume of protein extract used in enzyme reaction (mL), where 0.1 mL protein extract was used in feed activity assay

3.1.4 Divide A590" by the sample dry weight (mg)
3.1.5 AC1 activity of the samples is defined as A590"/mg flour(feed)
3.1.6 Calculate the average and standard deviation

3.2 Positive control activity calculation
3.2.1 Calculate the average of the A590 readings of three positive controls
3.2.2 Subtract the average blank A590 reading from the average positive A590(pcA590)
3.2.3 Divide pcA590 by the amount of positive protein added in the reaction

Where:

0.05mL of 500ng/mL (500 ppb) was added in the reaction, 0.05 x 500 = 25ng = 0.000025mg

3.2.4 Enzyme activity of the positive control (A590/mg protein) equals to pcA590/0.000025

Protocol 2. GraINzyme® AC1 Glucanase ELISA Assay

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model:TP650-9)
1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)
1.1.3. IKA® Tube Mill 100 Control (b) (4)
1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43
1.1.5. Eppendorf benchtop clinical centrifuge Model 5810 or equivalent
1.1.6. Laboratory gloves
1.1.7. Pipette tips (b) (4)

Calibration date:_____ Expiration Date:_____

1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20pl, 100pl and 200pl)

Calibration Date: _____ Expiration Date:_____

1.1.9. 4.5 ml TallPrep Tubes (b) (4)
1.1.10. 50mL Reagent Reservoirs (b) (4)
1.1.11. 96-well Plate Covers (b) (4)
(b) (4)
1.1.12. 96 well block, 2ml (b) (4)
1.1.13. Flat bottom 96 well plate (b) (4)
1.1.14. Water bath
Manufacturer: Benchmark
Model number: B2100-12
Serial number: MBG6120U-148
1.1.15 1L glass bottles
1.1.16 500ml glass bottles
1.1.17 Aluminum foil
1.1.18 Timer
1.1.19 Bench top centrifuges
Manufacturer: Spectrafuge
Model number: C2400-B
Serial number: D812826 and D812827
1.1.20 Permanent markers for labeling
1.1.21 Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492
1.1.22 Metal spatula
1.1.23 pH 4.0 Calibration Solution (b) (4)
1.1.24 pH 7.0 Calibration Solution
1.1.25 pH 10.0 Calibration Solution
1.1.26 Balance

Manufacturer: Mettler
Model number: PM4000
Serial number: F80835

1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number: Synergy HT
Serial number: 191338

1.1.28 pH meter
Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 ml conical centrifuge tube (b) (4)

1.1.30 Micro centrifuge tubes. 2.0 ml (b) (4)

1.1.31 PETG Flasks, 250 mL (b) (4)

1.1.32 Anthos Fluido 2 Microplate Washer (b) (4)

1.1.33 Titer plate shaker (b) (4)

1.2 Reagents

1.2.1 200mM Phosphate Stock Buffer, pH 6.5 (2L)

1.2.1.1 1M monosodium phosphate, monohydrate (ACROS Organics, AC389870000, MW 119.98g/mol)
Weigh out 119.98 g and dissolve in distilled water to a final volume of 1L.

1.2.1.2 1M disodium phosphate (Sigma, S5136, MW 141.96g/mol).
Weigh out 141.96 g and dissolved in distilled water to a final volume of 1L.

1.2.1.3 Mix 170ml monosodium phosphate, monohydrate with 230ml disodium phosphate, making pH 6.5. Adjust volume to 2L.

1.2.1.4 Filter buffer and store the buffer at 4C.

1.2.2 Extraction Buffer: 100mM NaPi, pH 6.5, 0.01% Tween 20 (1L)

1.2.2.1 Mix 500ml Phosphate stock buffer with 500ml deionized water

1.2.2.2 Add 0.1ml of concentrated Tween-20

1.2.3 QuantiPlate™ Kit for Beta-Glucanase (b) (4)
Including Enzyme Conjugate, Substrate, Stop Solution

1.2.4 Phosphate buffered saline (PBS), pH 7.4 (b) (4)
Dissolve 1 pack of PBS in 1L deionized water as protein diluent and plate wash buffer

2. Experimental Procedures

2.1 Sample Preparation, Protein Extraction and Extract Dilution

2.1.1 Sample Preparation

2.1.1.1 Grind negative corn seeds for LOD detection in IKA Tube Mill, 130,000 rpm for 1 minutes, which produces 94% product with <0.6 mm grind size

2.1.1.2 Grind corn grains in the Knife Mill to less than 1.0 mm grind size for feed preparation

2.1.1.3 Grind feed samples in the Knife mill to less than 1.0 mm grind size for protein extraction and activity test

2.1.2 Protein extraction

2.1.2.1 Extract protein by adding 5 volumes Extraction Buffer which is pre-warmed at 60°C (e.g. 100 ml to 20 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers at 60°C, 250 rpm for 1 hour. After one hour extraction, vortex containers for 10 seconds before centrifuging the aqueous extract at 16,000 g for 10 min. The supernatant is saved for the ELISA assay.

2.1.3 Protein dilution for ELISA assay

2.1.3.1 Dilute purified AC1 protein to make a standard curve.

The purified protein is diluted to 0.12, 0.23, 0.45, 0.90, 1.24, 1.40, 1.60, and 1.80 ng/mL or ppb in PBS buffer.

2.1.3.4 Protein extract was diluted with PBS diluent to allow sample ODs to fall on the standard curve.

2.2 ELISA Assay

GraINzyme AC1 protein ELISA assay procedure refers to (b) (4) (b) (4) for GraINzyme® Beta-Glucanase™ protocol. (b) (4)

2.2.1 Allow ELISA plate/strips to warm up to ambient temperature before removing the plate/strips from bag with desiccant, it takes about 30 minutes.

2.2.2 Calculate the buffer needed for each step, and dispense the buffer into a sample tray 20 minutes before usage. For example, 100 ul of each buffer (i.e. Enzyme Conjugate Buffer, Substrate, and Stop Solution) is required for one sample well at each step. If a test including 80 protein extracts and 16 (2x8) protein standards, at least 10 mL buffer is required. Make sure to keep the Substrate buffer in dark by covering the buffer tray with aluminum foil.

2.2.3 Organize all protein standards, sample extracts, and pipettes so that the next step (2.2.4) can be performed in 5 minutes or less. Multi-channel pipettes are used for all samples and reagents additions.

2.2.4 Add 100ul of non-diluted or diluted protein extract to an ELISA plate. Record the sample IDs.

2.2.5 Add 100 ul of protein standards on the ELISA plate. Mix thoroughly for 30 seconds.

2.2.6 Seal wells with a plastic wrap to prevent evaporation and incubate for 15 minutes at ambient temperature.

2.2.7 After incubation, remove the plastic wrap. Place the plate on the microplate washer. Perform four washes (300 pL/well). After the wash, aspirate the liquid.

2.2.8 Add 100ul Enzyme Conjugate to all wells. Mix thoroughly for 30 seconds.

2.2.9 Seal wells with the plastic wrap and incubate for 45 minutes at ambient temperature.

2.2.10 Wash the plate as in Step 2.2.7.

2.2.11 Add 100ul Substrate to all wells. Mix thoroughly 30 seconds.

2.2.12 Seal wells with the plastic wrap and incubate for 15 minutes at ambient temperature.

2.2.13 Add 100ul Stop Solution to each well and mix thoroughly by pipette up and down a few times. Using new pipette tips for each row of samples. Need to finish this step for all the samples in as short time as possible (about one minute).

2.2.14 Read absorbance of the plate within 5 minutes after addition of the Stop Solution. Read absorbance at 450 nanometers (A450) with a reference wavelength of 640 nanometers (A640).

3. Results Calculation

3.1 Subtract A640 value of each sample or protein standard from their corresponding A450 value (A450').

3.2 Make average of A450' from two buffer blank (blank_A450').

3.3 Subtract blank_A450' from A450' of each sample or protein standard (A450").

3.4 Make average of A450" from two standards of the same concentration.

3.5 Graph the concentration (ng/mL) vs the average A450" of each protein standard on linear scales. Apply a quadratic curve fit.

3.6 Calculate the concentration of each protein extract from the quadratic equation:

$$Y \equiv ax^2+bx+c$$

Where,

Y is the A450" of a sample protein extract

a, b, c is numerical coefficent of the quadratic curve

X is the concentration of AC1 detected in the sample extract

3.7 Multiply each concentration (X) by dilutions.

3.8 Multiply sample concentration (ng/mL) by the extraction volume to obtain AC1 protein content in the extract (ng).

3.9 Divide sample AC1 content (ng) by the sample dry weight (mg).

3.10 **AC1 content in the sample is expressed as ug per gram of dry weight.**

Protocol 3. GalNzyme® AC1 Glucanase Activity Unit Assay Protocol

Introduction: To define activity units for AC1, a two-step approach will be applied.

Step one: enzymatic hydrolysis of barley-β-glucan by AC1 at 80°C to release reducing sugars such as glucose. When protein extract is appropriately diluted, the initial velocity will be detected within 40min of the reaction. It is important to test units from multiple dilutions of protein extract at 40min for a sample whose activity is unknown. If the activities from different dilutions are comparable, subsequent tests can be done at one or two dilutions.

Step two: detection of reducing sugars from the enzymatic hydrolysis and from a set of glucose standards with BCA reagent at 80°C.

One unit (U) of AC1 activity equals 1 μmol/min glucose reducing equivalents released from 1% barley-β-glucan at 80°C, pH6.5

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model:TP650-9)

1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)

1.1.3. IKA® Tube Mill 100 Control (b) (4)

1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43

1.1.5. Eppendorf benchtop clinical centrifuge Model 5810 or equivalent

1.1.6. Laboratory gloves

1.1.7. Pipette tips (b) (4)

(b) (4)

Calibration date:_____ Expiration Date:_____

1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20μl, 100μl and 200μl)

Calibration Date: _____ Expiration Date:_____

1.1.9. 4.5 ml TallPrep Tubes (b) (4) (b) (4))

1.1.10. 50mL Reagent Reservoirs (b) (4)

1.1.11. 96-well Plate Covers (b) (4)

(b) (4)

1.1.12. 96 well block, 2ml (b) (4)

1.1.13. Flat bottom 96 well plate (b) (4)

1.1.14. Water bath

Manufacturer: Benchmark

Model number: B2100-12

Serial number: MBG6120U-148

1.1.15 1L glass bottles

1.1.16 500ml glass bottles

1.1.17 Aluminum foil

1.1.18 Timer

1.1.19 Bench top centrifuges

Manufacturer: Spectrafuge

Model number: C2400-B
Serial number: D812826 and D812827

1.1.20 Permanent markers for labeling

1.1.21 Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492

1.1.22 Metal spatula

1.1.23 pH 4.0 Calibration Solution (b) (4)

1.1.24 pH 7.0 Calibration Solution

1.1.25 pH 10.0 Calibration Solution

1.1.26 Balance
Manufacturer: Mettler
Model number: PM4000
Serial number: F80835

1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number: Synergy HT
Serial number: 191338

1.1.28 pH meter
Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 ml conical centrifuge tube (b) (4)

1.1.30 Micro centrifuge tubes, 2.0 ml (b) (4)

1.1.31 PETG Flasks, 250 ml (b) (4)

1.1.32 Heat Block
Manufaturer: VWR Scientific
Model number: 12621-084 or equivalent
Serial number: 2174

1.2 Reagents

1.2.1 200mM Phosphate Stock Buffer, pH 6.5 (2L)

1.2.1.1 1M monosodium phosphate, monohydrate (ACROS Organics, AC389870000, MW 119.98g/mol)
Weigh out 119.98 g and dissolve in distilled water to a final volume of 1L.

1.2.1.2 1M disodium phosphate (Sigma, S5136, MW 141.96g/mol).
Weigh out 141.96 g and dissolved in distilled water to a final volume of 1L.

1.2.1.3 Mix 170ml monosodium phosphate, monohydrate with 230ml disodium phosphate, making pH 6.5. Adjust volume to 2L.

1.2.1.4 Filter buffer and store the buffer at 4C.

1.2.2 Protein extraction Buffer: 100mM sodium phosphate, pH 6.5, 0.01% Tween 20 (1000ml)

1.2.2.1 Mix 500ml Phosphate stock buffer with 500ml deionized water

1.2.2.2 Add 0.1ml of concentrated Tween 20

1.2.3 Barley-β-glucan, low viscosity ((b) (4))

1.2.4 0.5M Hydrochloride (b) (4)

1.2.4.1 Take 4.132ml of concentrated HCl (12.1N) to a beaker

1.2.4.2 Add 95.868ml of distilled water to adjust volume to 100ml

1.2.5 Pierce BCA Protein Assay Kit

(b) (4)

1.2.6 D-glucose standard (Acros Organics, 170080010)

1.2.6.1 100mM glucose stock: 180.2mg/10mL prepared in extraction buffer (100mM sodium phosphate, pH 6.5, 0.01% Tween 20)

1.2.6.2 Dilute 100mM glucose to 10mM, 1mM, 0.8mM, 0.6mM, 0.4mM, 0.2mM, 0.1mM, and 0.05mM in the extraction buffer

1.2.6.3 Store glucose standard in 500ul aliquots at -20C

Glc stock used for dilution (mM)	Glc stock added (ul)	Extraction Buffer added (ul)	Final Glc (mM)	Final vol (ul)
100	200	1800	10	2000
10	200	1800	1	2000
10	160	1840	0.8	2000
10	120	1880	0.6	2000
10	80	1920	0.4	2000
10	40	1960	0.2	2000
10	20	1980	0.1	2000
1	100	1900	0.05	2000

2 Experimental Procedures

2.1 Mill grain or feed to less than 1mm grind size

2.2 Extract protein from samples by adding 5 volumes Extraction Buffer which is pre-warmed at 60°C (e.g. 100 ml Extraction Buffer to 20 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers at 60°C, 250 rpm for 1 hour. After one hour extraction, vortex container for 10 seconds before centrifuging the aqueous extract at 16,000 g for 10 min. The supernatant is saved for the activity unit assay

2.3 Protein extract dilution

2.3.1 Dilute the extracts by 40-, 160-, 240-, 320 or 360- fold as needed (feed sample might need dilution at 2- to 10- fold)

2.3.2 Dilute purified protein 100-fold as a positive control for assay validation

2.3.2.1 Purified AC1 protein (200,000ppb) was stored in 50mM MES, 150mM sodium chloride, pH6.3 buffer plus 40% glycerol at -20C

2.3.2.2 Make 100-fold dilution to 2000ppb

2.4 Barley-β-glucan digestion by AC1

2.4.1 Turn on a water bath before beginning procedure to allow it to come up to 80C.

2.4.2 Turn on the heat block to 80C.

2.4.3 Weigh out Barley-β-glucan based on the number of reactions, e.g. 10 samples, 4 dilutions for each sample will need total 40 reactions. Each reaction needs 5mg substrate, therefore, 40*5=200 mg of Barley-β-glucan is required. Need to weigh out at least 220mg to prevent the loss of substrate solution during pipetting. Dissolve the substrate with the extraction buffer (100mM NaPi pH 6.5, 0.01% Tween 20) at 80C. Substrate must be prepared before the test.

Barley-β-glucan weighed _____ mg

Extraction buffer added _____ml

Extraction buffer (ml) = (0.45 * weighed mg of barley-β-glucan)/5 = 0.09 * weighed mg of barley- β-glucan

2.4.4 Dissolve substrate by incubating at 80C in the water bath, occasionally vortexing until the substrate is fully dissolved.

2.4.5 Cluster tubes are used for 40min endpoint activity unit assay.

2.4.6 Test up to 9 samples, a negative control, and two positive controls in one cluster tube block.

2.4.6.1 In the block of cluster tubes : dispense 450ul of the substrate into tubes of A2 to D12. Record the name of samples and their dilutions which will be dispensed into the corresponding well in Table 1. These rows will serve as the reaction.

2.4.6.2 Add 450ul of the extraction buffer (no substrate added) to each control tubes from rows E2 to H12 corresponding to each sample tube. This will serve as the blank to correct protein content detected by BCA method for each reaction. Record the name of the sample in the corresponding tube on Table 1

2.4.7 Add 50ul of diluted sample extract including the negative control to each blank tube first, E2 to H12 excluding E12 and F12.

2.4.8 Add 50ul of diluted protein extract including the negative control to each tube containing substrate (reaction), A2 to D12 excluding A12 and B12.

2.4.9 Add 50ul of 2000ppb purified protein into E12 and F12, then A12 and B12

2.4.10 Cover the tubes with Corning™ Storage Mat III, use the Corning Storage Mat Applicator to seal tubes tightly. Shake the plate at a low speed to mix well.

2.4.11 Place the block in the water bath. Add a weight on the top of the cluster tube to prevent the mat from popping up during 80°C incubation period.

2.4.12 Start timer for the 40min enzyme hydrolysis

2.4.13 Pool enough 0.5N HCL to a sample tray

2.4.14 At 40 min of incubation, take the cluster tube block out from the water bath, remove the mat, and add 100ul 0.5N HCL to each well, starting from wells A2 to H12 using a multichannel pipette. Pipette up and down three times. Keep the block on ice.

Table 1 (Deep-well plate)

	1	2	3	4	5	6	7	8	9	10	11	12	Dilutions	
A		Sample_1									Neg. Ctr	Pos. Ctr	160	
B		Sample_1									Neg. Ctr	Pos. Ctr	240	
C		Sample_1									Neg. Ctr		320	
D		Sample_1									Neg. Ctr		360	Reaction
E		Sample_1									Neg. Ctr	Pos. Ctr	160	
F		Sample_1									Neg. Ctr	Pos. Ctr	240	
G		Sample_1									Neg. Ctr		320	
H		Sample_1									Neg. Ctr		360	Blank

2.5 Using BCA method to quantify glucose reducing equivalents

2.6.1 Prepare BCA reagent: mixing reagent A with reagent B by 50:1 (i.e. 50ml reagent A mixed with 1ml reagent B)

2.6.2 In a microplate, to make a glucose standard curve, dispense 75ul of the extraction buffer in the first well of column 1 (A1), 75ul of each glucose standard in the remaining wells of column 1 (one set of glucose standards). Refer to Table 2.

2.6.3 To detect reducing sugars in sample reactions, add 50ul of extraction buffer to the plate (A2 to H12) using multichannel pipette, and then remove 25ul of each reaction and blank from the tubes on Table 1 to the microplate, starting from A2, until all reactions and blanks were added to the microplate. Record the sample ID in each well in Table 2.

2.6.4 Add 175ul BCA reagent to each well with samples or standards using a multichannel pipette, pipetted up and down to mix.

2.6.5 Place the plastic sealed plate to an 80°C heat block for 10 minutes

2.6.6 After 10 minutes incubation, cool the plate on ice for 10 minutes

2.6.7 Spin the plate for 5 minutes to bring down the condensate

2.6.8 Record absorbance at 560nm (A560) for all the samples and controls in the microplate

Reaction/Ctr (ul)	25
Extraction buffer (ul)	50
BCA (ul)	175
Total (ul)	250

Glucose Std (ul)	75
BCA (ul)	175
Total (ul)	250

Table 2 Flat-bottom microplate sample layout (BCA test)

	1	2	3	4	5	6	7	8	9	10	11	12	Dilutions	
A	Extt buffer	Sample_1									Neg. Ctr	Pos. Ctr	160	
B	0.05	Sample_1									Neg. Ctr	Pos. Ctr	240	
C	0.1	Sample_1									Neg. Ctr		320	
D	0.2	Sample_1									Neg. Ctr		360	Reaction
E	0.4	Sample_1									Neg. Ctr	Pos. Ctr	160	
F	0.6	Sample_1									Neg. Ctr	Pos. Ctr	240	
G	0.8	Sample_1									Neg. Ctr		320	
H		Sample_1									Neg. Ctr		360	Blank

3. Activity unit calculation

One unit (U) of AC1 activity equals 1 µmol/min glucose reducing equivalents released from 1% Barley-β-glucan at 80°C, pH 6.5 using the BCA method.

3.1 Glucose standard curve:

3.1.1 Correct absorbance glucose standard (A560) by subtracting the absorbance values from the reagent blank (Column 1: A1) for each of the standards

3.1.2 Plot the absorbance at 560nm versus the concentrations of glucose

3.1.3 Calculate the "best fit" line through the dataset using linear regression.

$Y = aX + b$

Where:

Y is the average corrected absorbance for each glucose concentration standard

X is concentration of each glucose standard

a is the slope

b is the intercept

3.1.4 Determine glucose reducing equivalents in each AC1/barley-β-glucan reaction:

3.1.4.1 Subtract the absorbance at 560nm of the sample blank from the absorbance of each corresponding sample reaction

3.1.4.2 Use the regression equation from the glucose standard to calculate the glucose content (umol) in the sample

3.1.4.3 Divide the amount of glucose (umol) released from barley-β-glucan at 40min reaction by 40 to determine the amount of reducing units produced per minute.

$A/40$ umol/min

Where:

A is the amount of glucose (umol) released from substrate at 40min

Unit = Dilution x (A/40)/gram of flour used for protein extraction

Where

Dilution = E/50 * D * 1 * 24

	Volume (ul)	Dilution
Sample was extracted in E ul buffer	E	

Protein extract was diluted by D-fold		D
50 ul of the protein extract was added to the reaction with Barley-beta-glucan (final vol. 500ul)	50	=E/50
Total volume of hydrolysate (ul)	500	
500ul hydrolysate were mixed with 100ul acid (600ul)	500	1
Total volume of the mixture with acid (600ul)	600	
25 ul of the above reaction (600ul) was added to the BCA test (final 250ul)	25	24

3.1.5 The final sample activity will be adjusted by subtracting the activity units of the negative control (protein extracts from the non-AC1-expressing grains) at the same dilution

3.1.6 Validate the assay using positive controls

3.1.6.1 Subtract the blank absorbance at 560nm (average absorbance of E12 and F12) from the absorbance of each reaction test (A12 or B12)

3.1.6.2 Use the regression equation from the glucose standard to calculate the glucose content (umol)

3.1.6.3 Divide the amount of glucose (umol) released from barley-β-glucan at 40 minutes reaction by 40 to determine the amount of reducing units produced per minute.

A/40 umol/minute

Where:

A is the amount of glucose (umol) released from substrate at 40 minutes by purified protein

Unit = Dilution x (A/40)/mg of protein in the assay

Where

Dilution = 24

mg of protein in the assay = 2000*(50/1000)/1000000

where

2000ng/ml was the concentration of the positive control used for the test

50ul of 2000ng/ml positive control was included in the reaction and blank

	Volume (ul)	Dilution
50 ul of positive control was added to the reaction with Barley-beta-glucan (final vol. 500ul)	50	
Total volume of hydrolysate (ul)	500	
500ul hydrolysate were mixed with 100ul acid (600ul)	500	
Total mix with acid (600ul)	600	
25 ul of the above reaction (600ul) was added to the BCA test (final 250ul)	25	600/25 =24

T-2

From: jim.ligon@agrivida.com
To: Tang, Lei
Cc: Wong, Geoffrey K
Subject: Re: Publication of AC1 poultry tolerance study
Date: Tuesday, August 27, 2019 1:04:35 PM

Hi Dr. Tang,

This paper was first published online in JAPR in February this year as you say. It took some time until it was published in the print version of JAPR as this was just released in the Sept. 2019 issue. It is the same paper as before but now it is also in the print version of the journal. Sorry for the confusion, I should have explained this to you before.

Many thanks,

jim

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
www.agrivida.com

jim.ligon@agrivida.com
919-675-6666

1023 Christopher Drive
Chapel Hill, NC 27517

On Aug 27, 2019, at 10:54 AM, Tang, Lei <Lei.Tang@fda.hhs.gov> wrote:

Dear Dr. Ligon,

Just to follow up with your email below. This paper was published online in the Journal of Applied Poultry Research on February 20, 2019, which was before the date of your submission (May 28, 2019). Thank you for letting us know that this paper is now in the September issue of the paper publication. Is there any changes in the content of the paper compared to the online publication in February? Please let me know.

Best regards,
Lei Tang, Ph.D.
Chemist

Center for Veterinary Medicine
Office of Surveillance and Compliance
Division of Animal feeds

U.S. Food and Drug Administration

Tel: 240-402-5922
lei.tang@fda.hhs.gov

<image013.png>

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From: jim.ligon@agrivida.com <jim.ligon@agrivida.com>
Sent: Monday, August 26, 2019 3:33 PM
To: Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>
Subject: Publication of AC1 poultry tolerance study

Hello Mr. Wong,

In our recent GRAS notice for the use of the AC1 Glucanase product in poultry (AGRN #31), we cited Broomhead *et al*. (2019) that describes the 10X tolerance study with poultry. This publication was in press at the time we submitted our notice but it has since published and I want to make you aware of this. It appears in the Journal of Applied Poultry Research, volume 28, pages 631-637. I have attached a copy of this publication for your information and use.

Best regards,

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
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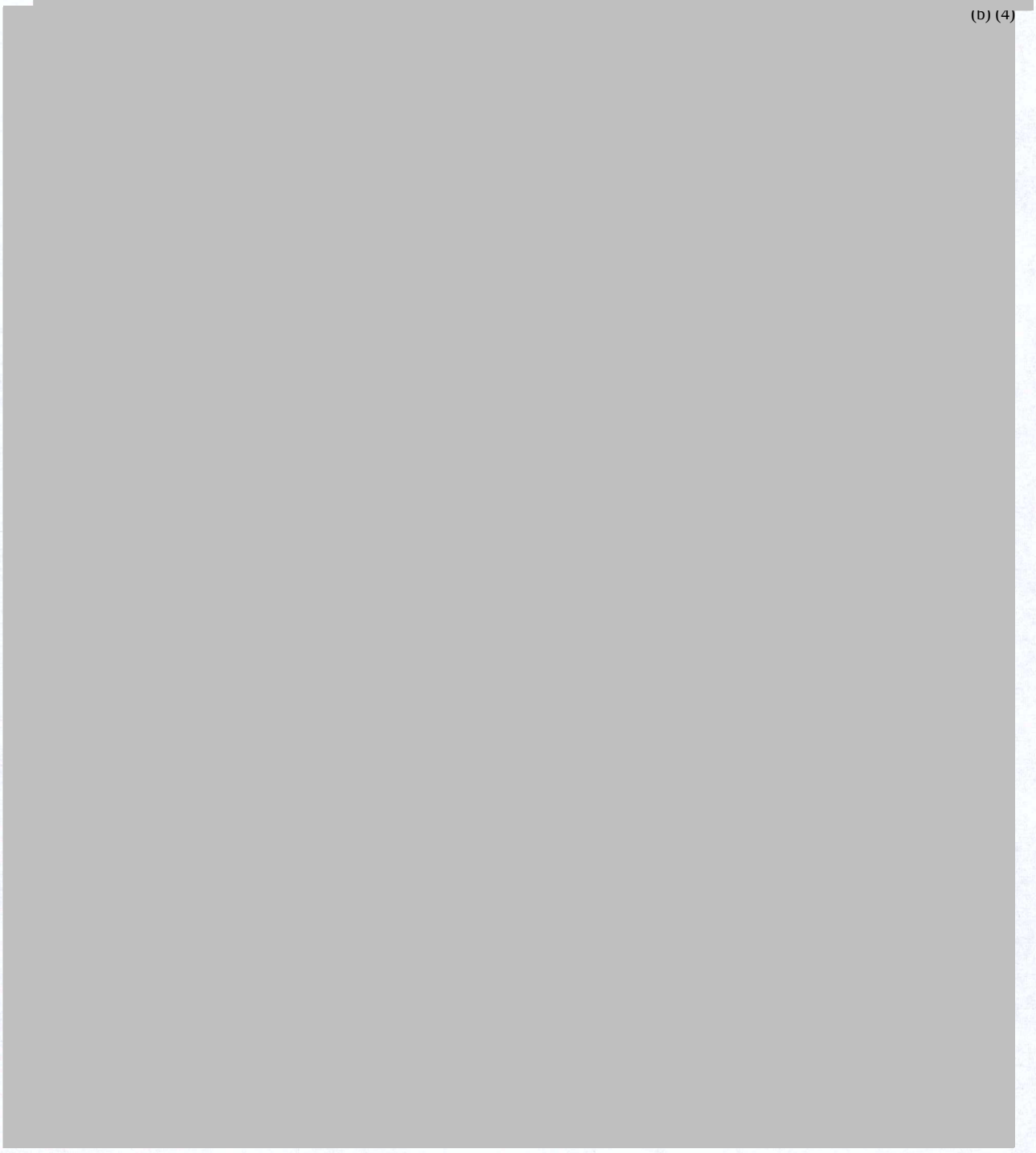
(b) (4)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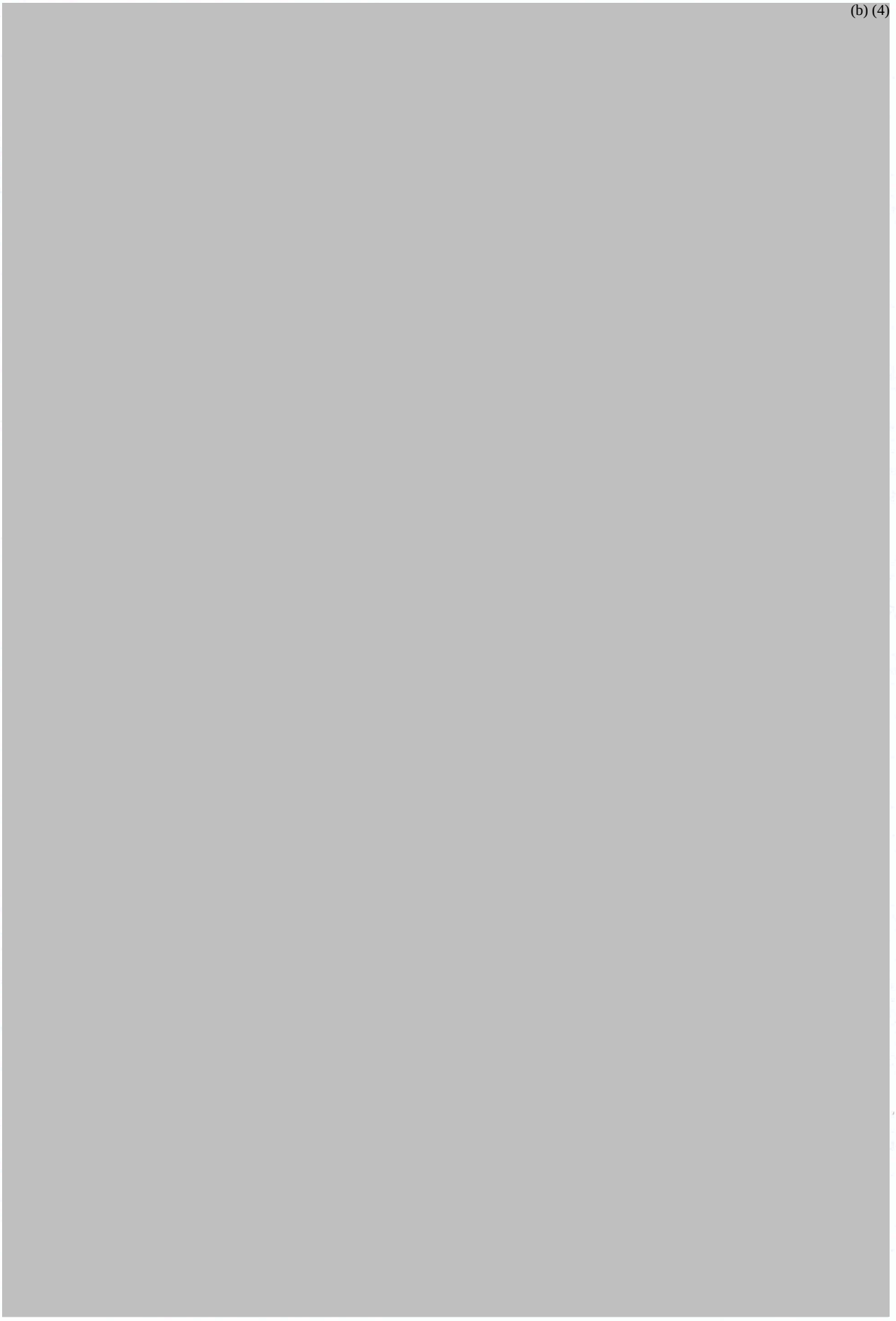
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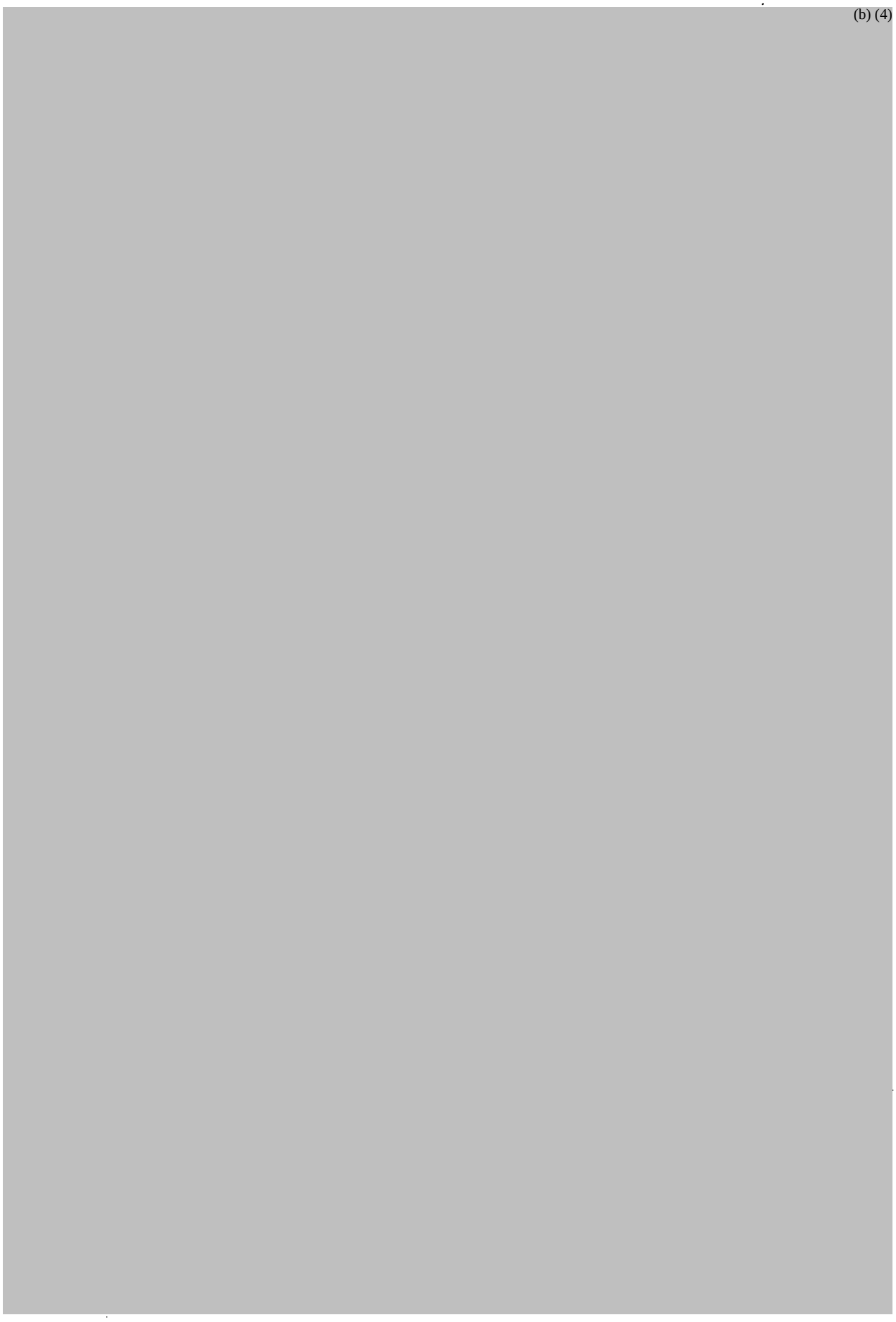
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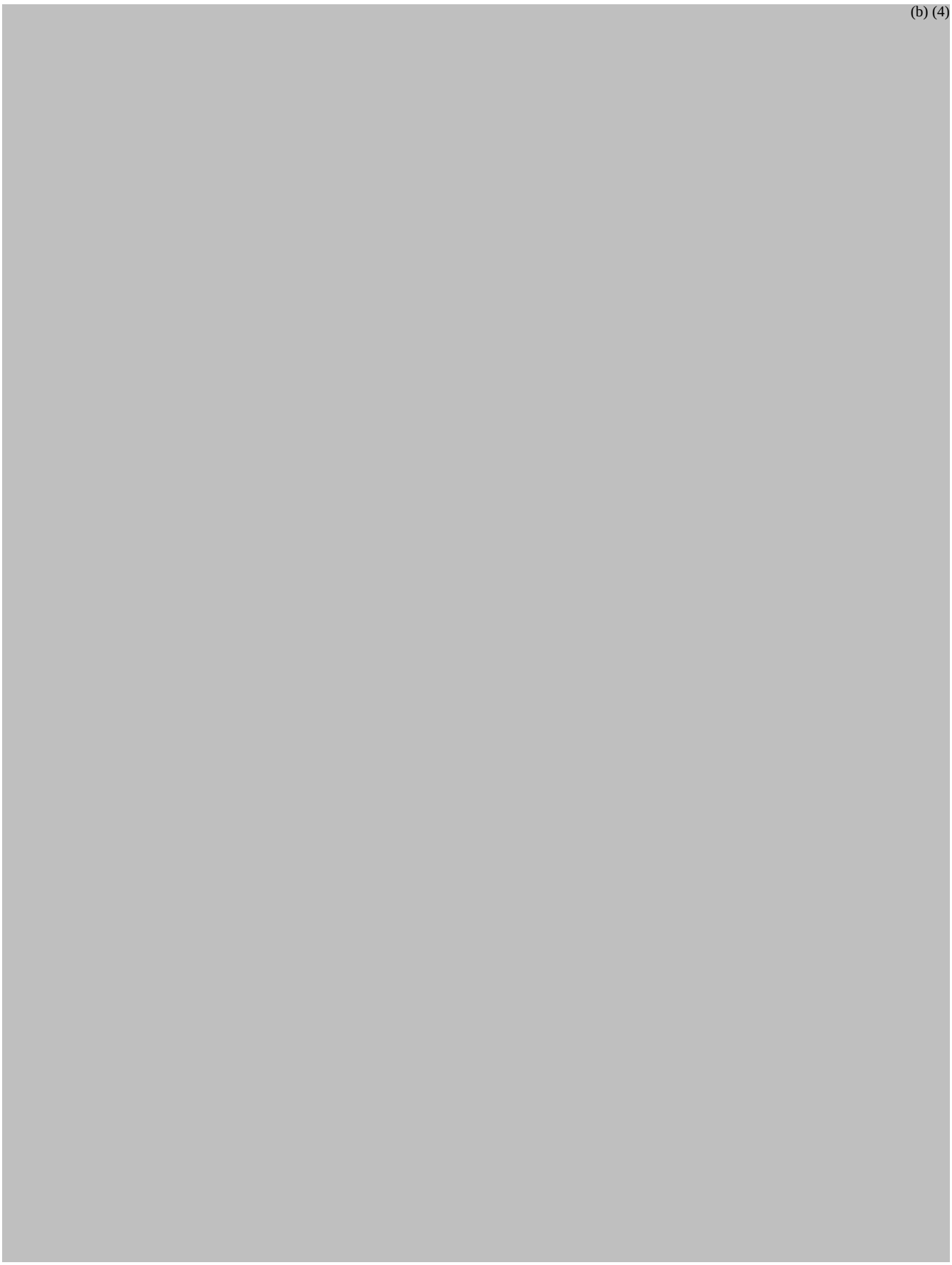
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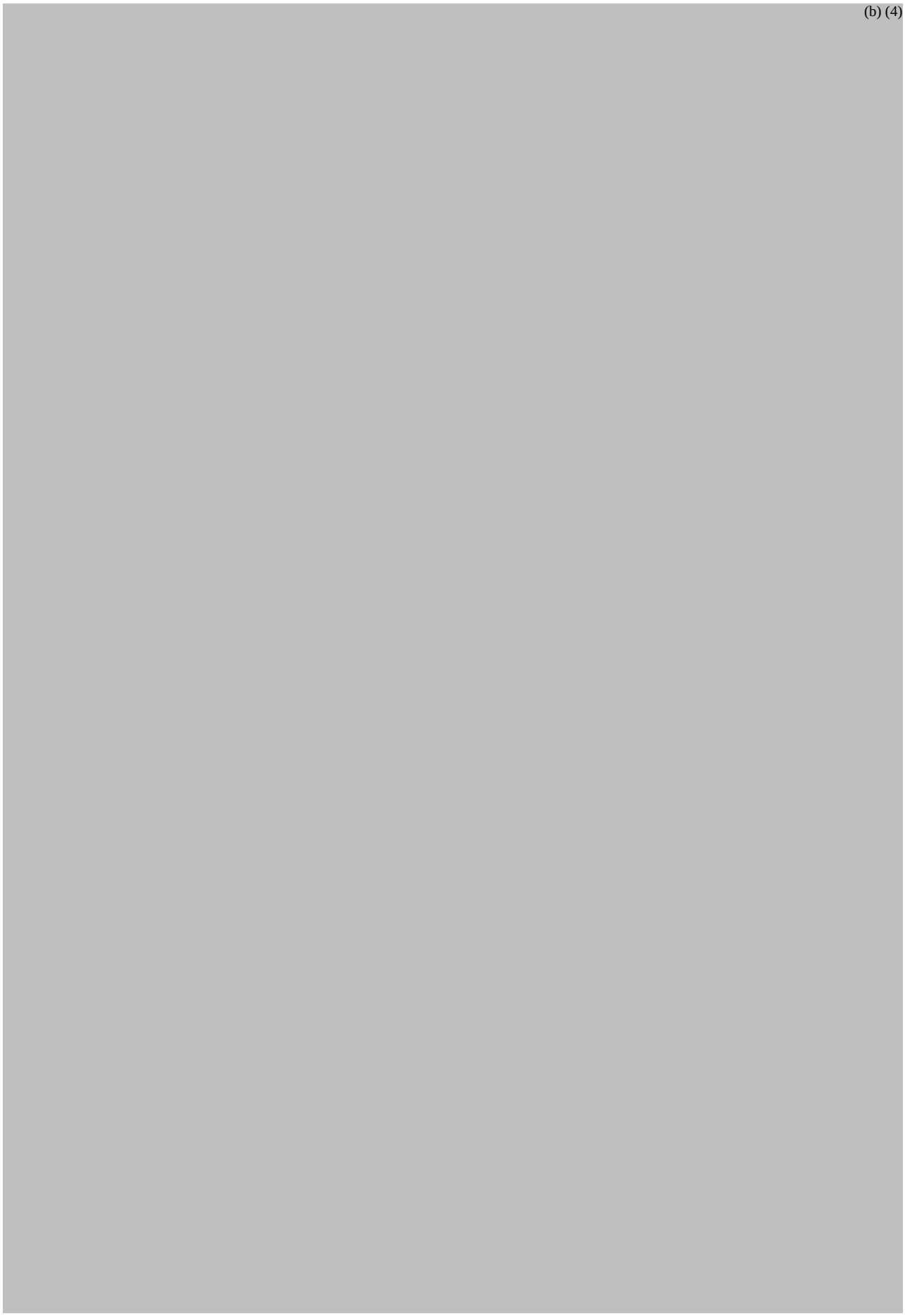
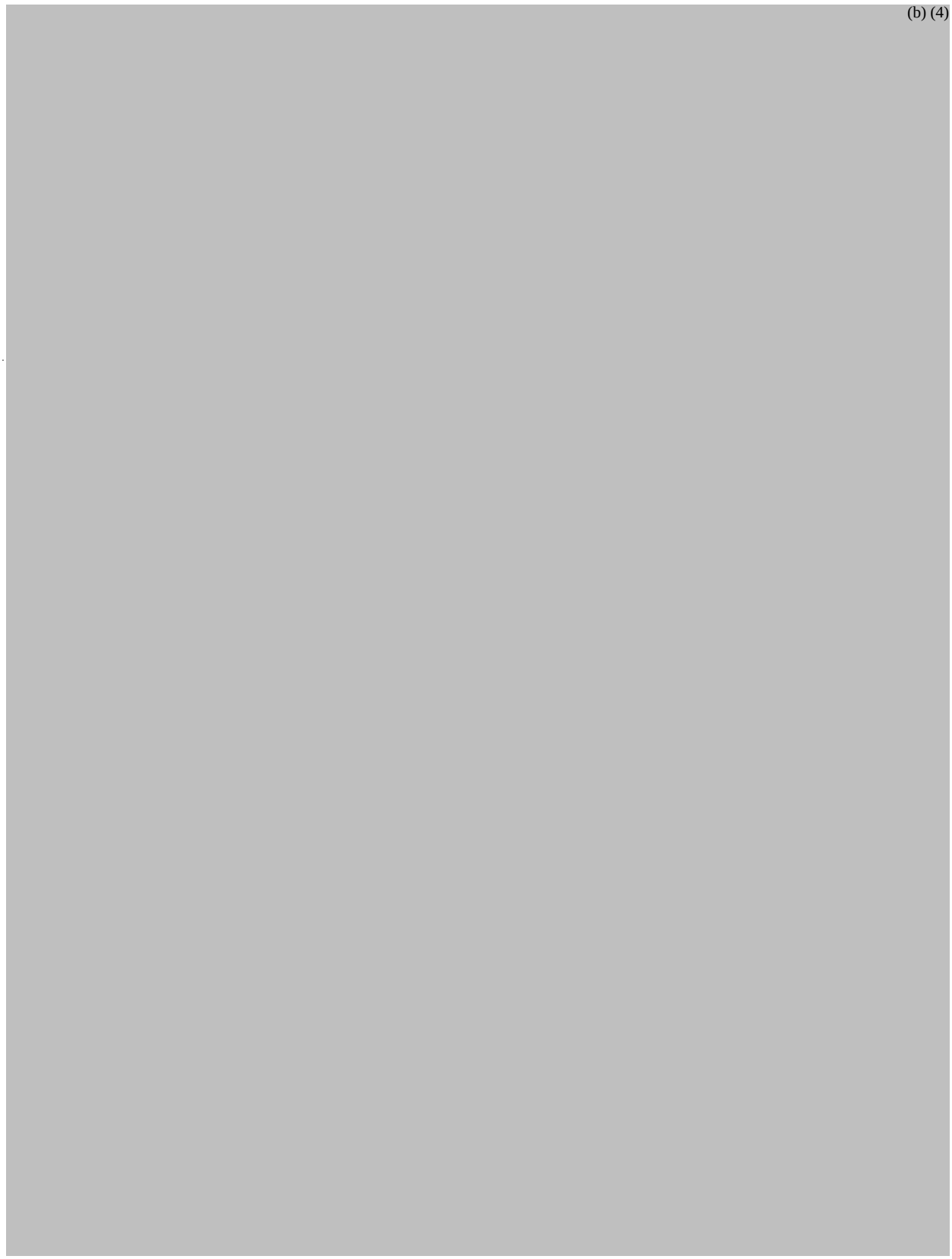

(b) (4)

(b) (4)

[redacted]


(b) (4)

T-4

From: jim.ligon@agrivida.com
To: Tang, Lei
Cc: Wong, Geoffrey K; Michael Raab R. Ph.D.; Phil Lessard
Subject: Amendment to AGRN 0031
Date: Wednesday, February 5, 2020 2:32:52 PM
Attachments: AGRN31 Amendment_5Feb20.pdf
Harper 2012 Bioinformatic analysis of T-DNAs.pdf
Lai 2005 Transposons and haplotype diversity in maize.pdf
Llaca 2011 Genome diversity in maize.pdf
Sandmeyer 2010 Retrotransposon near FG259 insertion site.pdf

Dear Dr. Tang,

We have completed an amendment to our GRAS Notice for the use of the Agrivida Glucanase (AC1) in poultry feed. This amendment contains responses to the issues and questions that were presented by CVM in our teleconference of 24 January, 2020. I have attached a copy of the amendment in PDF for your information. There are four new literature reports that are cited in the amendment and so I am also attaching copies of these to facilitate your further review. None of the information contained in this amendment is considered by Agrivida, Inc. to be confidential business information.

If you or others on the review team have further questions related to AGRN 0031, please feel free to contact me.

Best regards,

Jim Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
www.agrivida.com

jim.ligon@agrivida.com
919-675-6666

1023 Christopher Drive
Chapel Hill, NC 27517

A thermotolerant β-glucanase feed enzyme expressed in *Zea mays*

AMMENDMENT TO GRAS NOTICE No. AGRN 000-031

Submitting Company:
Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801

Please address correspondence related to this submission to:

James M. Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
1023 Christopher Drive
Chapel Hill, NC 27517

Tel: 919-675-6666
Email: jim.ligon@agrivida.com

February 4, 2020

Introduction

The FDA Center for Veterinary Medicine (CVM) is reviewing GRAS Notice No. AGRN 000-031, submitted by Agrivida, Inc. for its GraINzyme® Glucanase product in May 2019. During the review, CVM has developed questions related to the GRAS notice. These questions were presented to Agrivida at a teleconference on January 24, 2020 and are contained in the minutes of the meeting. Agrivida has carefully considered each of the questions from CVM and has formulated responses to address each question. These responses are contained in this amendment to the GRAS Notice No. AGRN 000-031. In this amendment, the issue raised by CVM is stated, followed by Agrivida's response.

1. Claims on the intended utility of the GraINzyme® Glucanase

Issue/question from CVM:
CVM noted that in Section 1.4 "Conditions of use of the notified substance", the intended utility of the GraINzyme® Glucanase is to "...increase the digestibility of feed containing soluble NSP." CVM informed Agrivida, Inc. that this claim for the utility of the enzyme is too broad and that it should be narrowed. Therefore, Agrivida, Inc. is amending the utility claim in Section 1.4 of the GRAS Notice to read as follows:

1.4 Conditions of use of the notified substance

This GRAS notice establishes the GRAS status of GraINzyme® AC1 Glucanase for use in the feed of poultry in order to decrease the viscosity of digesta in the intestinal tracts of poultry consuming feeds containing high amounts of soluble NSP. The recommended inclusion rate of the GraINzyme® AC1 Glucanase in poultry feed is 200 to 500 glucanase activity units (U) per kg of feed.

Related to the above issue, CVM requested that the discussion in the GRAS Notice about the positive impact on animal performance such as body weight, feed conversion ratio, etc. from the addition of the GraINzyme® AC1 Glucanase to poultry feeds containing high amounts of NSP should be cited as support for the safety of the AC1 Glucanase rather than its functionality or utility. The poultry feeding studies from which this data were generated are discussed in Sections 6.3.3 and 6.3.4 of the Notice. In this amendment we are providing a modified version of Sections 6.3.3 and 6.3.4 in which the word "functionality" that appeared in these sections in the original Notice have been replaced with the word "safety". The amended version of Sections 6.3.3 and 6.3.4 are included below:

6.3.3 Positive impact of AC1 Glucanase on poultry performance

The safety of the AC1 Glucanase in poultry feed is supported by the demonstration that its inclusion in feed has a positive impact on various

aspects of animal performance. Ayres *et al.* (2018) report that the inclusion of AC1 Glucanase in a high-NSP diet at 400 U/kg increased the weight gain after 14 days to be equivalent to that of the animals that received the low-NSP PC diet. It was also demonstrated in this study that the FCR of the birds receiving the high-NSP diet with 400 U/kg AC1 Glucanase was equivalent to that of the control group receiving the low-NSP diet without AC1 Glucanase.

In a similar study, Jacek *et al.* (2018) reported a positive impact on animal performance criteria due to the inclusion of AC1 Glucanase in the feed. In this study 728 Cobb 500 male broilers were randomly assigned to seven treatments with 13 replicates per treatment. The treatments consisted of a PC that received a standard diet, a NC that was formulated to contain 132 kcal less energy than the PC diet, and five treatments that received the NC diet amended with either 5, 50, 100, 250, or 500 U/kg AC1 Glucanase. After 16 days, measurement of body weight, FCR, and ileal digestibility of energy (IDE) measurements were made. Jacek et al. (2018) reported that inclusion of the AC1 Glucanase at 100 and 250 U/kg resulted in a significant increase ($p<0.01$) in body weights compared to both the PC and NC groups. In addition, the inclusion of AC1 Glucanase to the NC diets at more than 100 U/kg resulted in a significant increase ($p<0.01$) in IDE and restored the IDE to be equivalent to that of birds that received the PC diet ($p<0.05$). These results are consistent with those reported by Ayres *et al.* (2018) and they provide further support of the safety of the AC1 Glucanase in poultry diets.

6.3.4 Conclusions on the Functionality of GraINzyme® AC1 Glucanase

The ability of the AC1 Glucanase to decrease the viscosity of the digesta in the GI tract of poultry when included in feeds that contain wheat and DDGS has been demonstrated by Ayres *et al.* (2018). This finding is supported by another study (Ayres et al., 2019) where the viscosity of wheat and DDGS containing feed were reduced by the addition of the AC1 Glucanase. Additional support of the safety of the AC1 Glucanse in poultry feeds comes from two studies, one reported by Jacek *et al.* (2018) in which the inclusion of the AC1 Glucanase in poultry feed was demonstrated to increase body weight and IDE and to improve FCR. Another study by Jacek *et al.* (2018) demonstrated improved weight gain and IDE in broiler chickens receiving a feed supplemented with AC1 Glucanase. Taken together, these studies provide clear confirmation of the safety of the AC1 GraINzyme® Glucanase in the feed of poultry.

2. Question regarding maize genomic sequence surrounding the T-DNA locus in Event FG259

Question #1:
CVM identified that the furthest upstream part of the right T-DNA border flanking sequence in Event FG259 does not map to the same genetic location as the more proximal sequence in the right border flank. CVM requested Agrivida, Inc. to explain this situation.

Agrivida response:
The right border (RB) T-DNA flank in event FG259 that was isolated and sequence characterized by Agrivida consists of (b) (4) bp maize genomic DNA. Two regions of distinct sequence identity to unrelated genomic regions in the maize genome can be identified within this DNA fragment. The (b) (4) nucleotides of the RB flank that are most proximal to the T-DNA right border are 98.39% identical to nucleotides (b) (6) of maize chromosome (b) (4) according to the BLAST analysis performed with that sequence on B73 maize reference genome (https://www.maizegdb.org/, RefGen_v4). This RB flank specific (b) (4) bp sequence is positioned (b) (4) bp upstream of nucleotides (b) (4) that have 99.56% identity to (b) (4) bp sequence isolated as the left border (LB) flank in FG259. The missing (b) (4) bp of the maize genomic DNA (chromosome (b) (4), nucleotides (b) (4) were displaced by T-DNA during its integration process. The (b) (4) nt RB flanking sequence that is more distal to the T-DNA has no sequence homology to the region of the B73 genome sequence that is immediately upstream of the (b) (4) bp sequence (nucleotides (b) (4)) on maize chromosome (b) (4). This part of the RB flanking sequence is highly repetitive in the maize genome and is 95.78% identical to a part of a predicted sequence that encodes exon-2 of a copia-type pol polyprotein ((b) (4), nucleotides (b) (4)). The copia-type pol polyprotein is one of the functional genetic elements characteristic to LTR retrotransposons (Sandmeyer and Clemens, 2010). The presence of a retroelement-specific sequence in the RB flank upstream of the (b) (4) bp RB flanking sequence (nucleotides (b) (4)) in FG259 is indicative of an earlier transposon activity event at this genetic location, which could have occurred in either the (b) (4) variety that was used for transforming (b) (4) into maize to generate FG259. It is known that many homologous chromosomal regions can differ significantly between maize varieties in their sequence or gene content and that transposable elements play an important role in creating intraspecific genome sequence diversity in maize (Lai *et al.*, 2005; Llaca *et al.*, 2011). Therefore, it is not unexpected that the reference genome (B73) and the variety that we transformed ((b) (4) differ at this location.

Question #2:
CVM proposed that Agrivida, Inc. should evaluate the sequence relationship between the T-DNA in FG259 event and the sequence in LB flank that is identical to the annotated gene (b) (4) in the maize genome. CVM asked us to address

potential concerns of T-DNA disrupting this predicted gene sequence and whether it might express problematic novel fusion proteins.

Agrivida response:
The uncharacterized gene sequence annotated as (b) (4) represents an mRNA transcript and is assigned to nucleotides (b) (4) of chromosome (b) (4) in the B73 maize genome (RefGen_v4). The (b) (4) sequence is deposited to the NCBI sequence database as accession No. (b) (4). The T-DNA insertion in FG259 displaced (b) (4) bp of the maize genomic DNA on chromosome (b) (4) with nucleotide coordinates (b) (4). Thus, it appears that the T-DNA integration in FG259 has occurred within the predicted (b) (4) mRNA transcript sequence truncating it by (b) (4) nucleotides at its (b) (4)' end. The predicted mRNA transcript in (b) (4) codes for a cDNA (b) (4) (b) (4)) with its ATG start codon beginning at nucleotide (b) (4) and terminating in stop codon TAA at the nucleotide (b) (4) on maize chromosome (b) (4). Based on these sequence data, we conclude that the T-DNA insertion on the maize chromosome (b) (4) in FG259 has occurred (b) (4) bp downstream of the cDNA stop codon in the (b) (4)' untranslated region of the (b) (4) transcript. The T-DNA insertion in FG259 does not disrupt the transcription from the cDNA open reading frame (ORF) in the predicted gene (b) (4). Based on our sequence analysis, we conclude that there are no apparent potential safety concerns that the T-DNA insertion would lead to a truncation of predicted protein encoded by this cDNA, affect the protein function, or form a new fusion protein. Furthermore, our analysis of ORFs of the length equal or greater than (b) (4) (as recommended by Harper *et. al*, 2012) on both DNA strands around the left T-DNA border junction site in FG259 did not reveal any inadvertently formed ORFs spanning the sequence junction between the left T-DNA border and the LB flanking maize genomic DNA sequence.

<u>Sequences relevant to Agrivida's response:</u>

(b) (4)

(b) (4)

(b) (4)

catcataaggcgtgatgaattcatagcgtcatggaatattatgaaatcacatgc

Using the (b) (4) sequence (highlighted in blue text, above) as a query in BLASTN search of Zea mays sequences in the NCBI database reveals sequence identity to a retroelement:

(b) (4)

(b) (4)

LB (b) (4) bp flanking sequence homologous to nucleotides (b) (4) on maize chromosome (b) (4):

(b) (4)

(b) (4)

Overview of the wild-type locus: Maize B73 chromosome (b) (4) wild-type sequence (nucleotides (b) (4)) that is homologous to the T-DNA locus site in FG259:

- Sequences presented in non-highlighted black text were derived from the B73 reference genome. Due to the presence of a copia-like pol element (at the point marked by the beginning of the gray shaded text), the non-highlighted sequences were not directly isolated from the RB flank of Event FG259.
- The right border (b) (4) bp flanking sequence (nucleotides (b) (4) - (b) (4)) is highlighted in gray color. This region is homologous to the sequences that were directly isolated from the RB flank of Event FG259.
- The (b) (4) nucleotides that were displaced by insertion of the T-DNA in FG259 are underlined.
- The left border (b) (4) bp flanking sequence is presented by green colored letters, and are homologous to the LB (b) (4) sequence that was directly isolated from the LB flank of Event FG259 (see above).
- The cDNA part of the predicted gene (b) (4) is *italicized* and its stop codon is highlighted in *red* color. This sequence is also shown in (b) (4) with corresponding highlighting (further below), followed by the sequence of the predicted mRNA (cDNA) from this gene. Note that the mRNA would be transcribed from the opposite strand, and transcription would proceed from right to left, with translation ending at the highlighted stop codon, before reaching the position where the T-DNA has inserted.

(b) (4)

(b) (4)

(b) (4)

(b) (4) sequences deposited to GenBank:

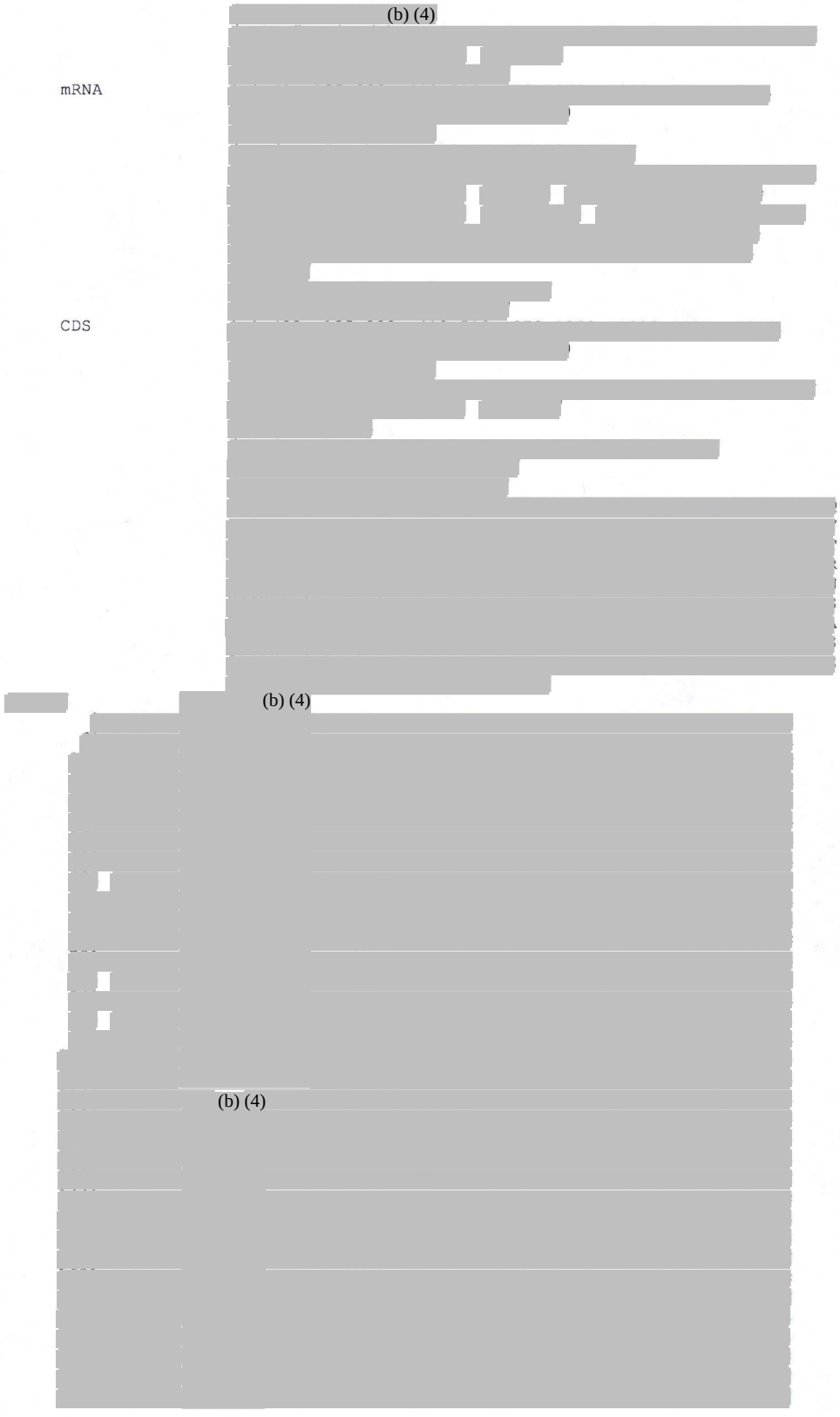
Note the sequences highlighted in green text or red shading are as described above.

Zea mays cultivar B73 chromosome (b) (4) B73 RefGen_v4, whole genome shotgun sequence
NCBI Reference Sequence: (b) (4)

(b) (4)

(b)
(4)

(b) (4)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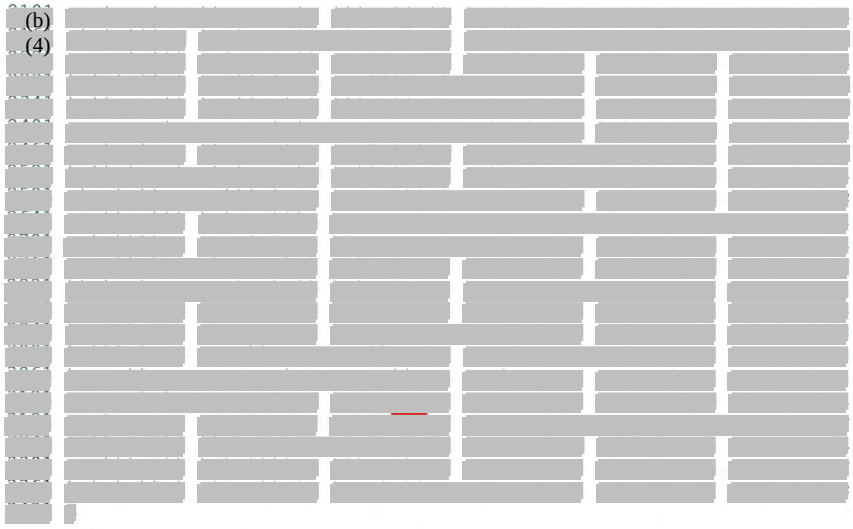

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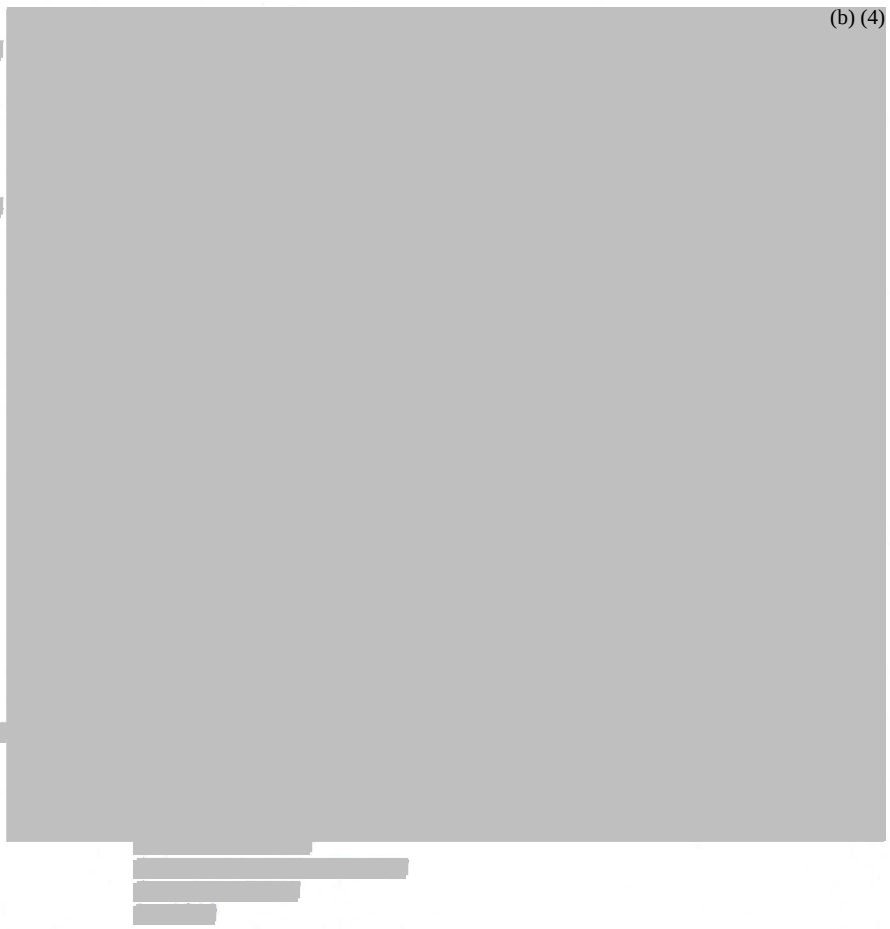

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Llaca, Victor, Matthew A. Campbell, and Stéphane Deschamps. 2011. "Genome Diversity in Maize." Journal of Botany 2011: 1–10. https://doi.org/10.1155/2011/104172.

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3. Differences in incubation times between the colorimetric and reducing sugar assays.

Issue/question from CVM:
Two protocols are presented for measuring the amount of glucanase activity in a given sample of grain or feed, a colorimetric and a reducing sugar (unit) assay. The colorimetric assay involves a 60-minute incubation in the presence of substrate, whereas the reducing sugar (unit) assay involves a 40 minute incubation in the presence of substrate. CVM inquired as to why the two assays have different incubation times. The shorter time used in the unit assay reflects the fact that the assay response becomes less linear, and therefore less reliable, with incubation times longer than 40 minutes. These differences in incubation times are normalized in the final calculations, as activity is expressed on a per-minute basis. To clarify the rationale for this difference, the introduction to Protocol 3 (page 132) is amended as follows:

> **Protocol 3. GraINzyme® AC1 Glucanase Activity Unit Assay Protocol**
> **Introduction:** To define activity units for AC1, a two-step approach will be applied.
> Step one: enzymatic hydrolysis of barley-β-glucan by AC1 at 80°C to release reducing sugars such as glucose. When protein extract is appropriately diluted, the initial velocity will be detected within 40 min of the reaction. It is important to test units from multiple dilutions of protein extract at 40 min for a sample whose activity is unknown. If the activities from different dilutions are comparable, subsequent tests can be done at one or two dilutions. Note: Incubation of the sample for longer than 40 minutes will give less reliable responses.

4. Enzyme assay validation table (Table 3) in Appendix 3.

Issue/question from CVM:
During the review of the glucanase assay validation that is presented in Appendix 3 of the Notice, CVM noted what appeared to be an error in the values for the concentration of the AC1 Glucanase enzyme that were used in the spiking and

recovery experiments with conventional corn that are presented in Table 3 on page 112. Indeed, upon close inspection of the table Agrivida, Inc. has confirmed that values in the table are incorrect. The correct values are presented in a revised Table 3 that appears below:

Table 3. Data from assay of AC1 Glucanase in the presence of ground WT corn.

AC1 spiked in extraction buffer	AC1 in activity assay	Extraction Buffer (A590)			Product Matrix (A590)			% Average Recovery
ppb	ng	Replicate_1	Replicate_2	Replicate_3	Replicate_1	Replicate_2	Replicate_3	(Matrix/Buffer)
0	0	0	0.002	0	0	0	0	0.00%
5	0.25	0	0.003	0.001	0	0	0	0.00%
10	0.5	0.003	0.003	0.004	0.009	0.01	0.011	300.00%
25	1.25	0.02	0.023	0.017	0.026	0.036	0.021	138.33%
50	2.5	0.029	0.03	0.027	0.045	0.047	0.047	161.63%
70	3.5	0.064	0.067	0.06	0.078	0.082	0.073	122.15%
125	6.25	0.102	0.109	0.11	0.115	0.138	0.147	125.09%
250	12.5	0.26	0.269	0.267	0.309	0.284	0.29	110.95%
500	25	0.527	0.599	0.575	0.655	0.61	0.591	109.30%
1000	50	1.276	1.341	1.311	1.406	1.428	1.391	107.58%
2000	100	2.525	2.724	2.499	2.793	2.666	2.454	102.27%
3000	150	3.888	Plateaued	Plateaued	Plateaued	Plateaued	3.682	94.70%

*Plateaued: these values were above the maximum detection threshold of the spectrophotometer.

5. Incorrect temperature for the extraction of AC1 Glucanase from ground maize grain and feed samples.

Issue/question from CVM:
In Sections 2.1 and 2.2 of Appendix 3 (page 111 of the Notice), the conditions that are used for extracting AC1 Glucanase from ground maize grain or feed were listed as "1 hour at 250 rpm and 80°C". CVM noted that since the protocol for extracting AC1 Glucanase from grain or feed samples (Protocol 1, section 2.1.2) calls for extraction at 60°C the reference to a temperature of 80°C in Appendix 3 seemed inaccurate. Agrivida, Inc. confirmed that the correct extraction temperature is indeed 60°C and so the citations of 80°C in Sections 2.1 and 2.2 of the original Notice are inaccurate. Therefore in Sections 2.1 and 2.2 of Appendix 3 the correct incubation is "1 hour at 250 rpm and 60°C."

The revised version of the first paragraph from section 2.1 is as follows:

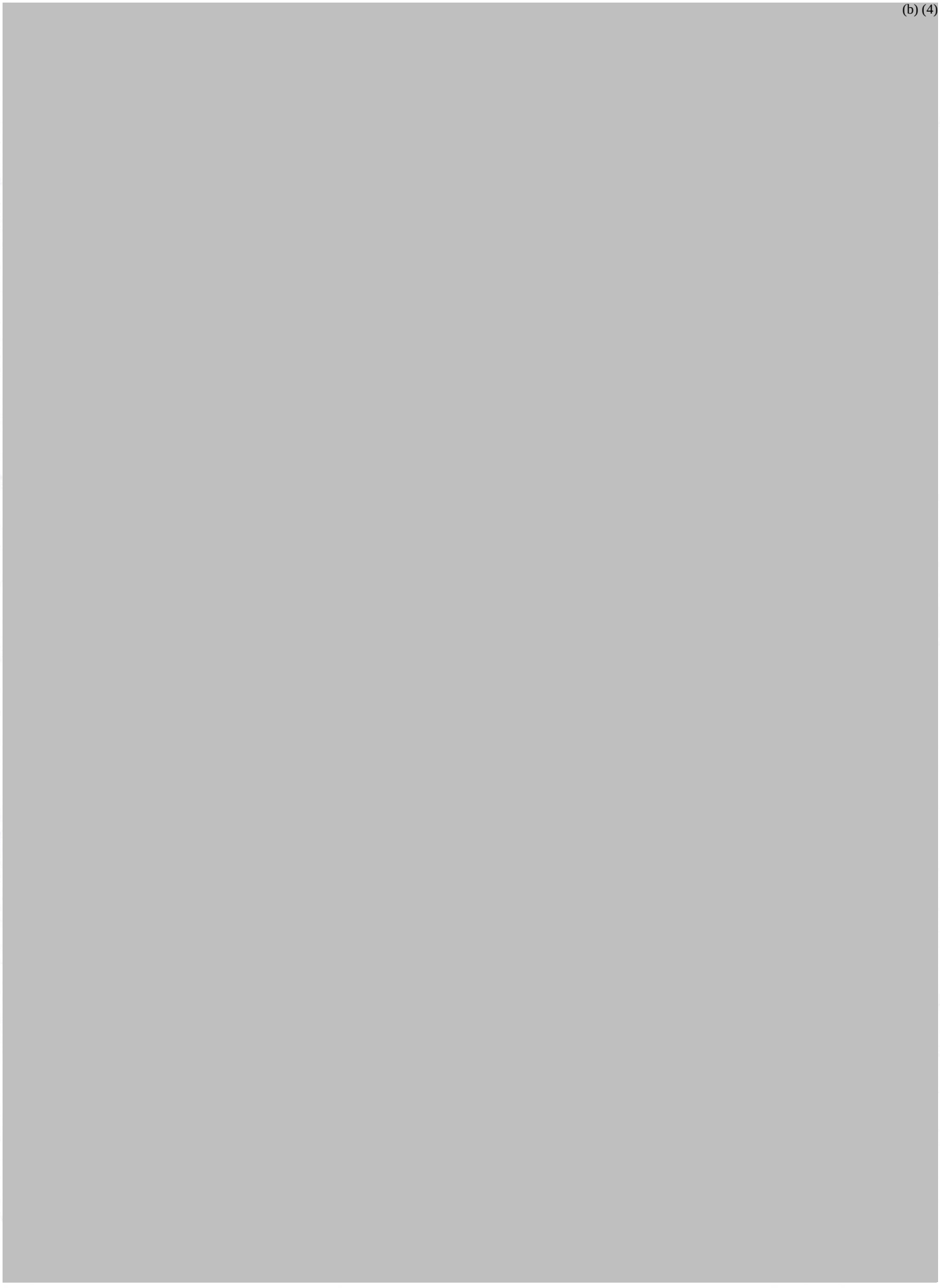
> 2.1 Mixing and Recovery of AC1 Glucanase activity from WT corn product
> Extraction buffer was spiked with AC1 Glucanase protein at concentrations of 5, 10, 25, 50, 70, 125, 250, 500, 1000, 2000 and 3000ppb. 2.5ml of each spiked extraction buffer was mixed with 0.5g of ground non-AC1 Glucanase corn in triplicate. The remaining spiked buffer and the buffer/corn mixes were placed on a temperature-controlled shaker, shaking for 1 hour at 250rpm and 60°C. After 1 hour shaking, the buffers and the buffer/corn mixes were removed from the shaker, and the buffer/corn mixes were

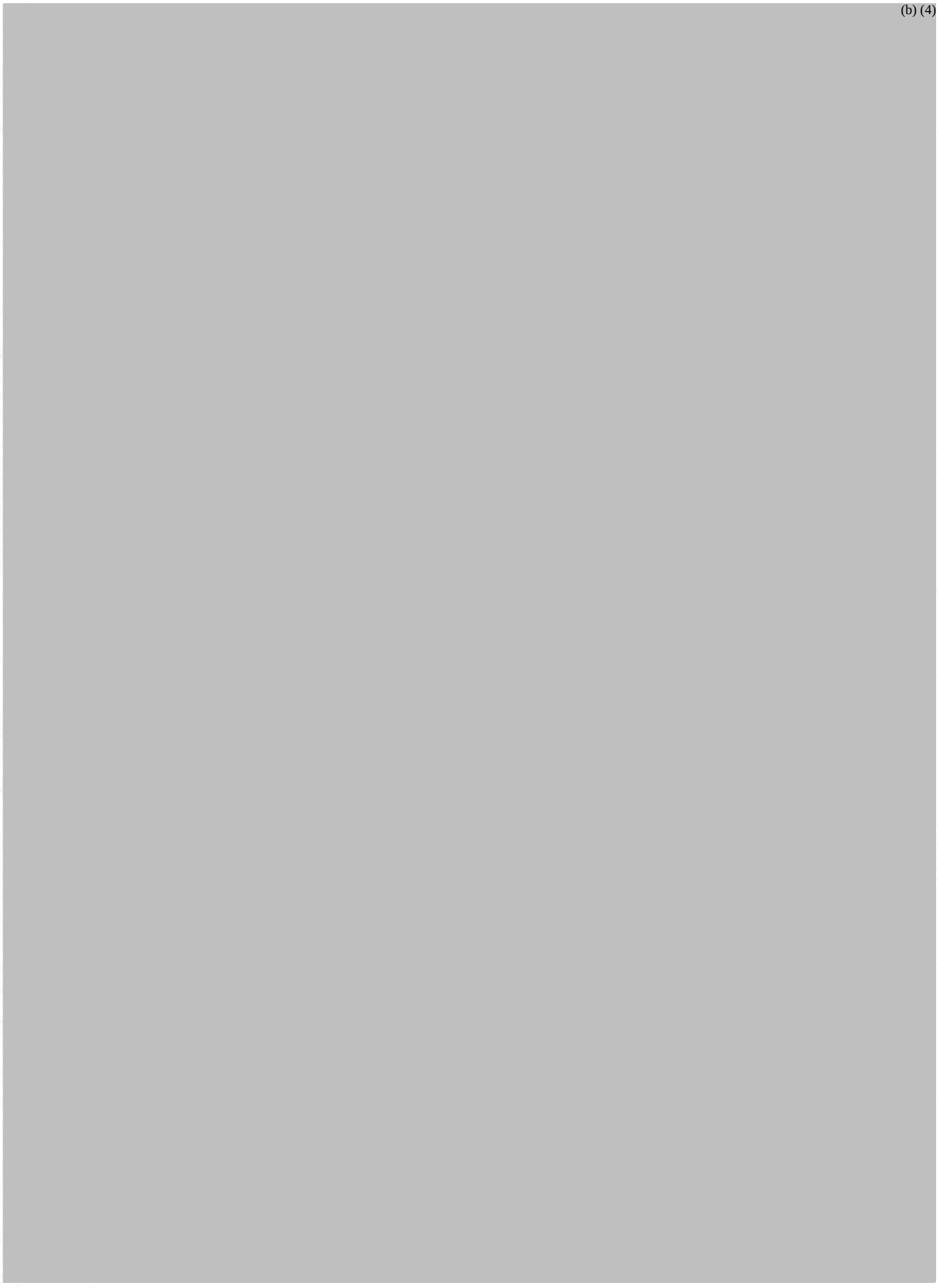
subjected to centrifugation. 50 µl of each buffer in triplicate or each supernatant (protein extract) from buffer/corn mixes was used for glucanase colorimetric assay. The amount of AC1 Glucanase protein used in enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 and 150 ng. The sample extraction and activity assay was carried out according to the standard protocol in Protocol I. AC1 Glucanase enzyme recovery is the average of triplicate assay activities from the product matrix relative to that from AC1 Glucanase spiked buffer.

The revised version of the first paragraph from section 2.2 is as follows:

2.2 Spiking and Recovery of AC1 activity from feed

To determine whether a complex feed mixture, such as that used in many poultry diets, might interfere with the sensitivity of the AC1 Glucanase assay, serial dilutions of the enzyme were again prepared in extraction buffer and mixed with samples of feed, then assayed for recoverable enzyme activity. For the feed sample, a corn/soybean feed (no AC1 Glucanase addition) was milled to less than 1.0 mm particle size. For the serial dilutions, extraction buffer was spiked with AC1 Glucanase protein at concentrations of 2.5, 5, 10, 25, 50, 70, 125, 250, 500, 1000, and 1500ppb, and 2.5ml of each buffer was mixed with 0.5g of feed sample in triplicate. The remaining spiked buffer and the buffer/feed mixes were placed on a temperature-controlled shaker, shaking for 1 hour at 250rpm and 60°C. 100ul of supernatant ("extract") was removed from each sample and was used in the standard assay as described in Protocol I. The amount of AC1 Glucanase protein used in enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 and 150ng, respectively. AC1 Glucanase enzyme recovery is the average of triplicate assay activities from the feed matrix relative to that from AC1 Glucanase spiked buffer.

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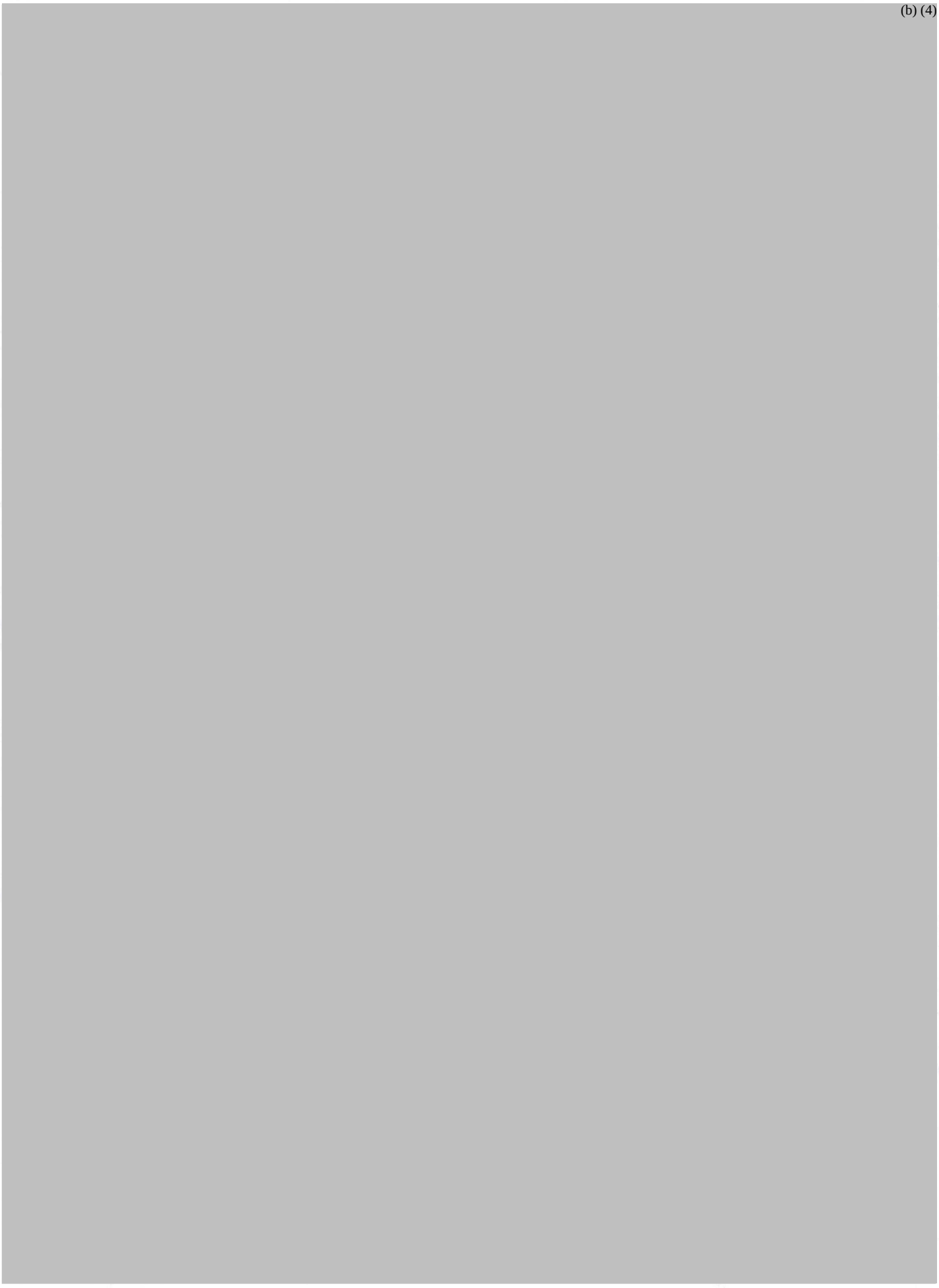
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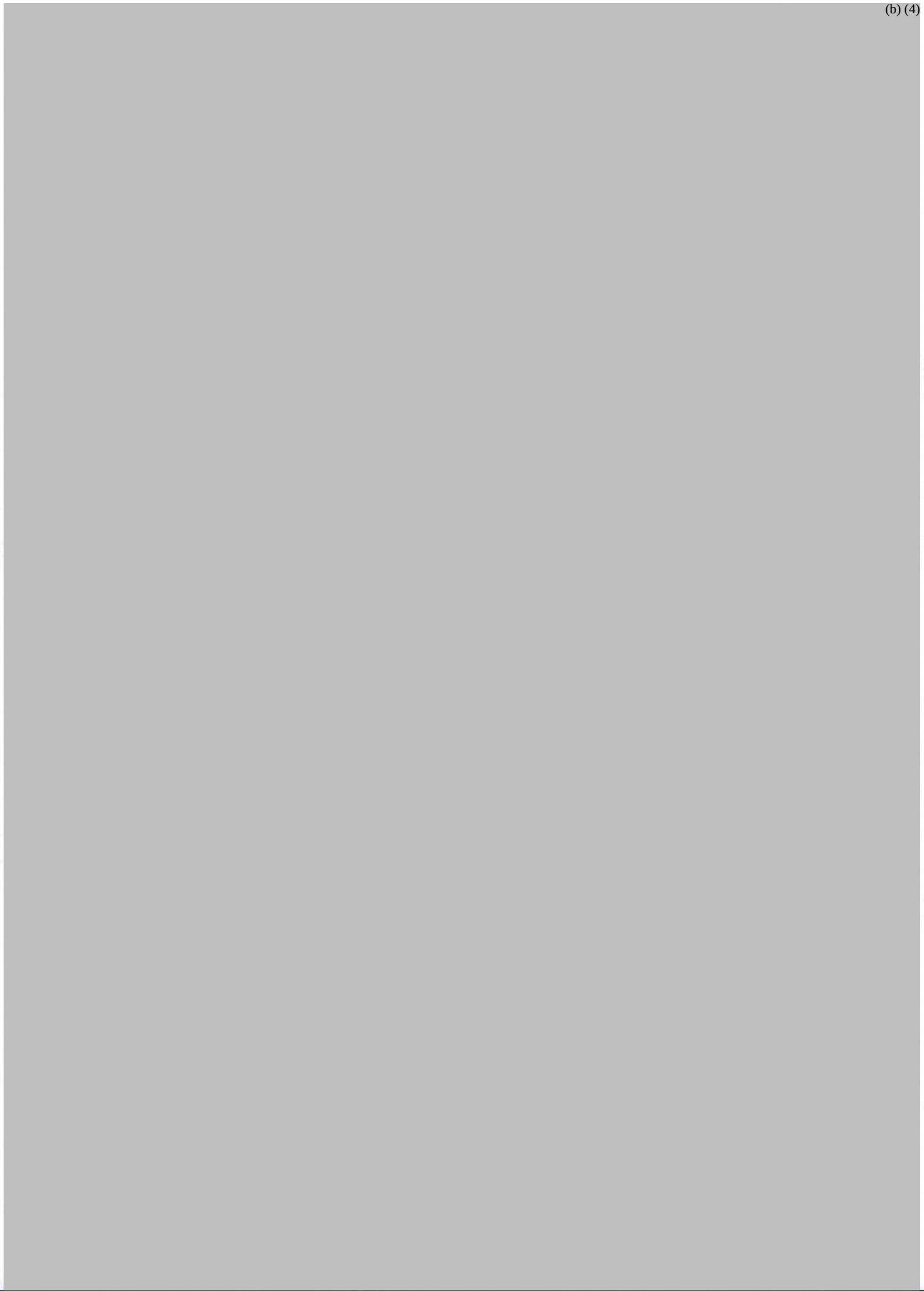

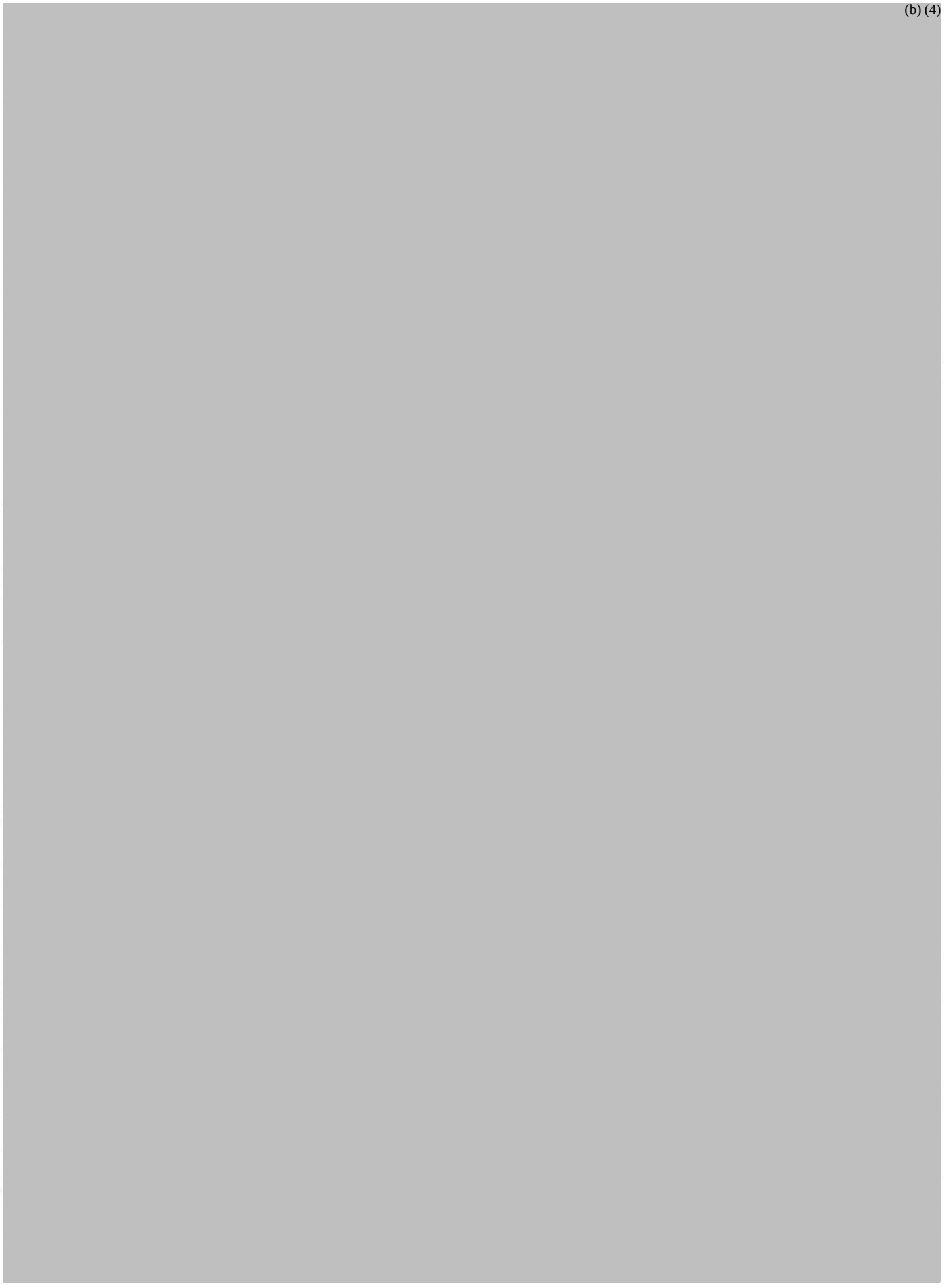
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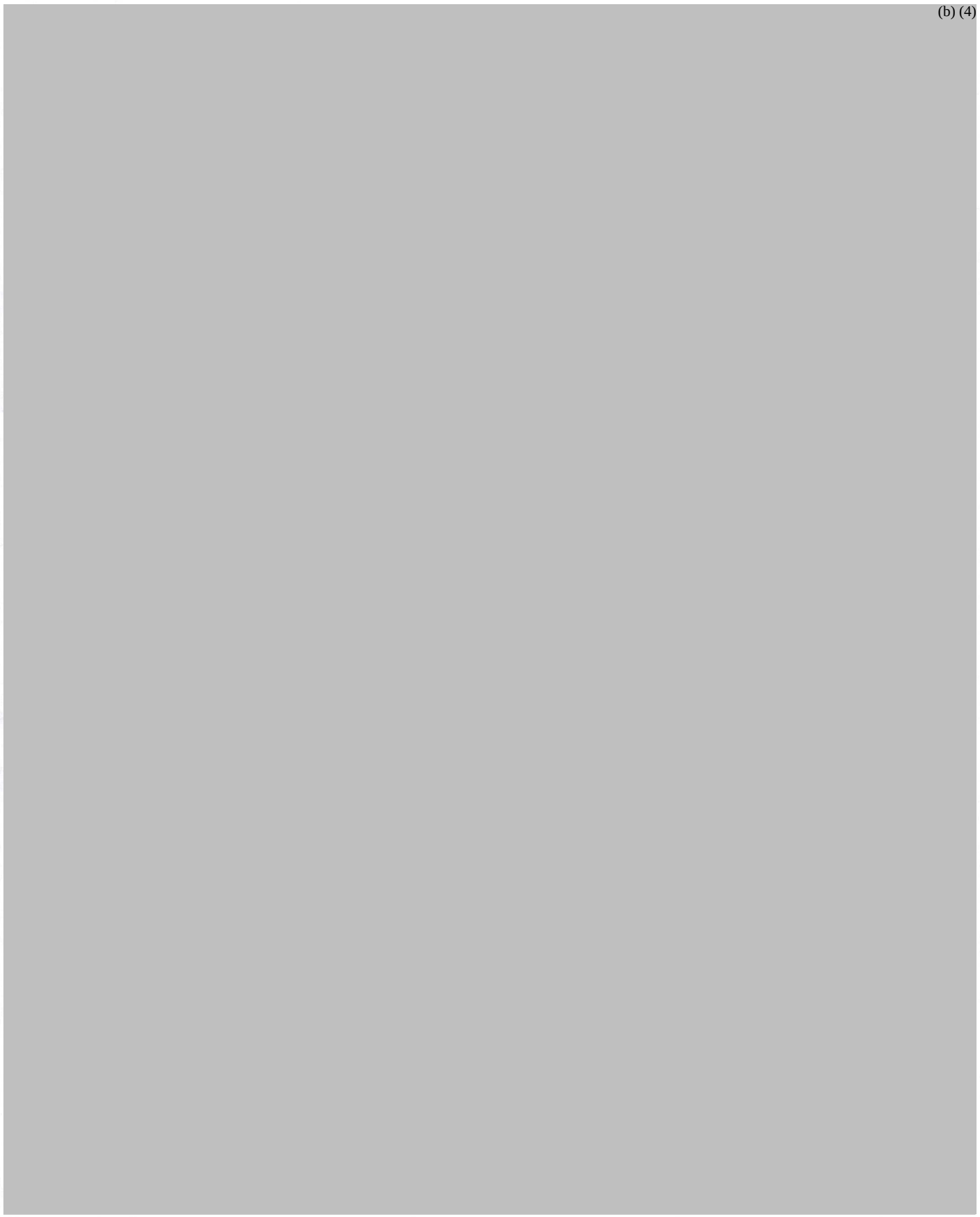
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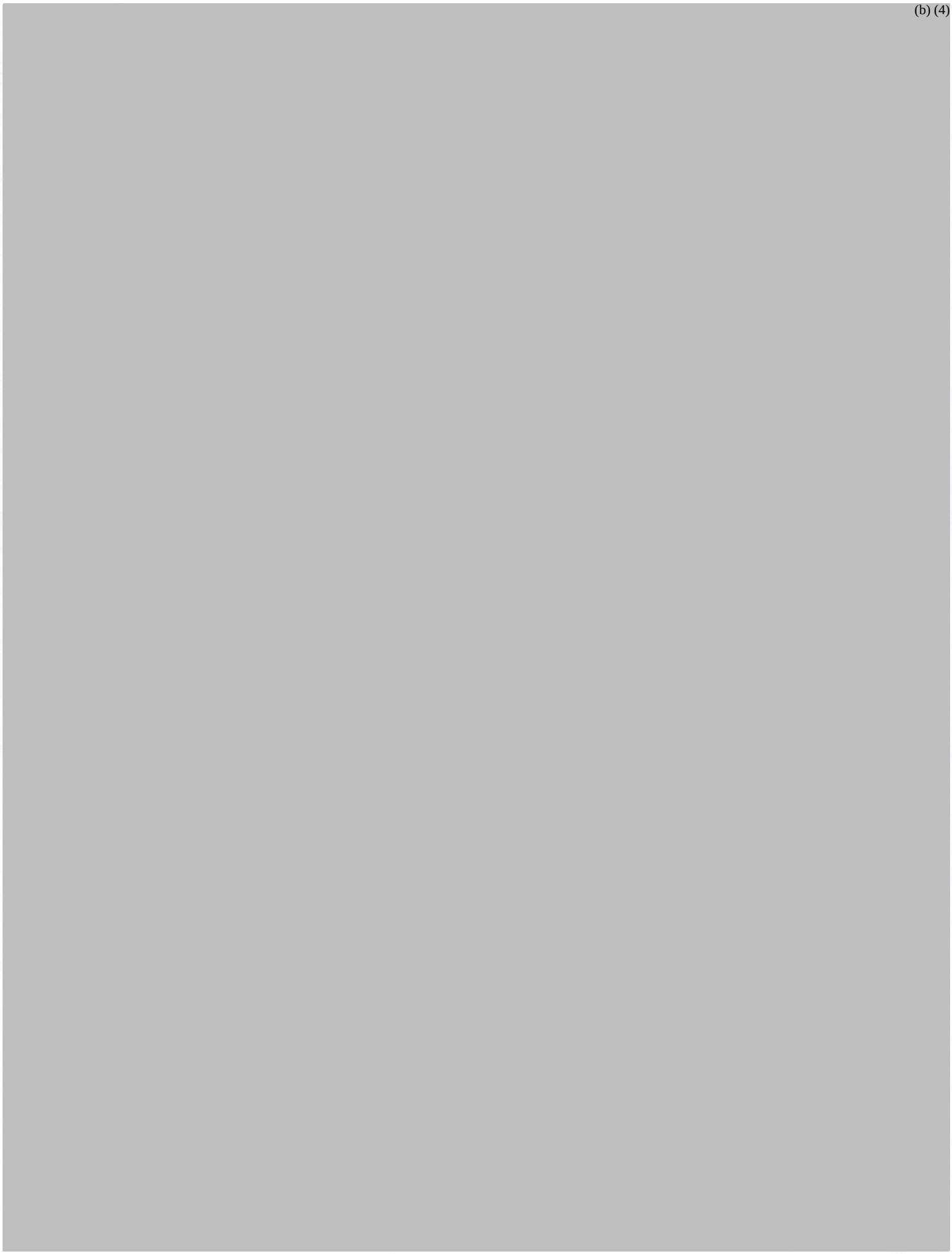

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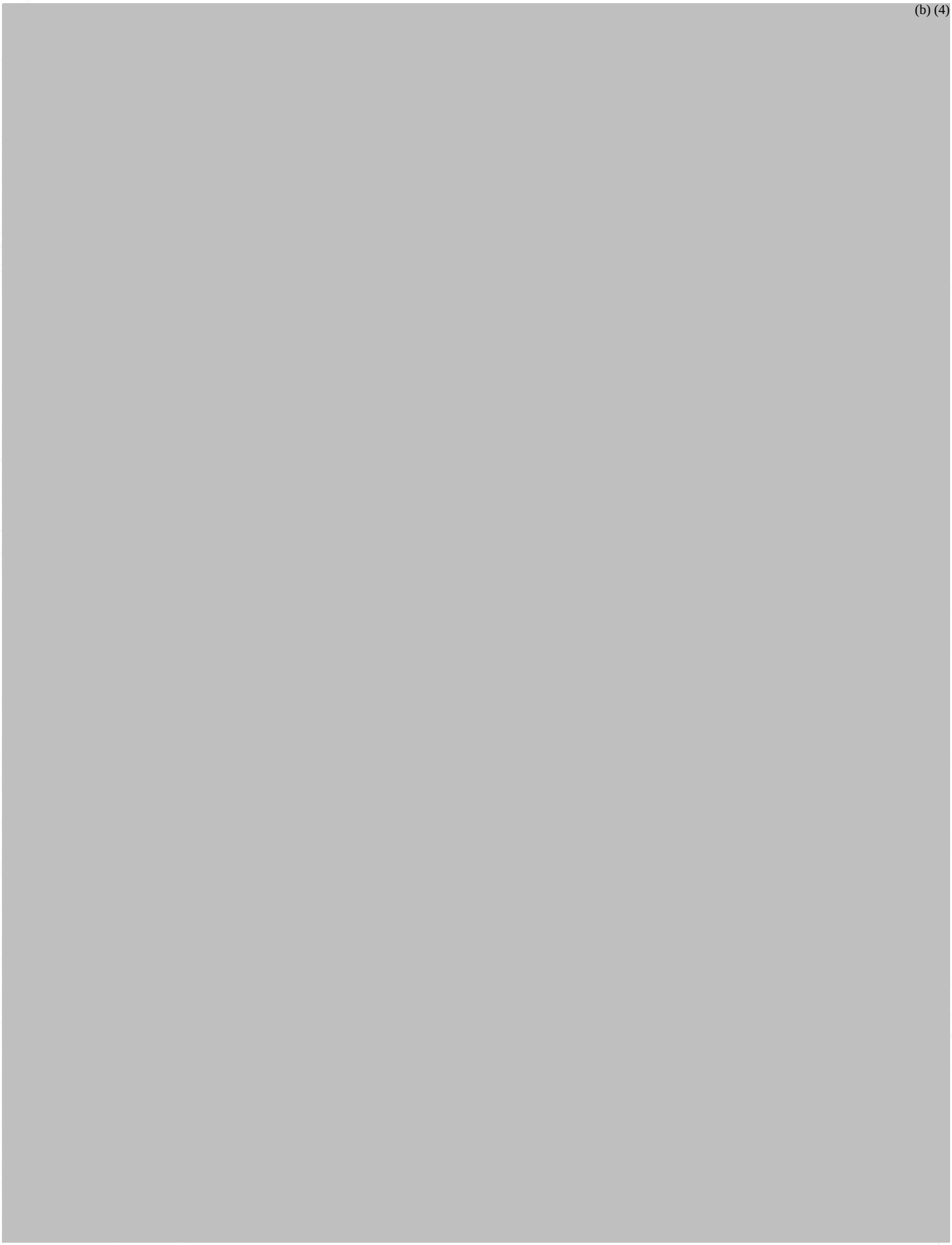
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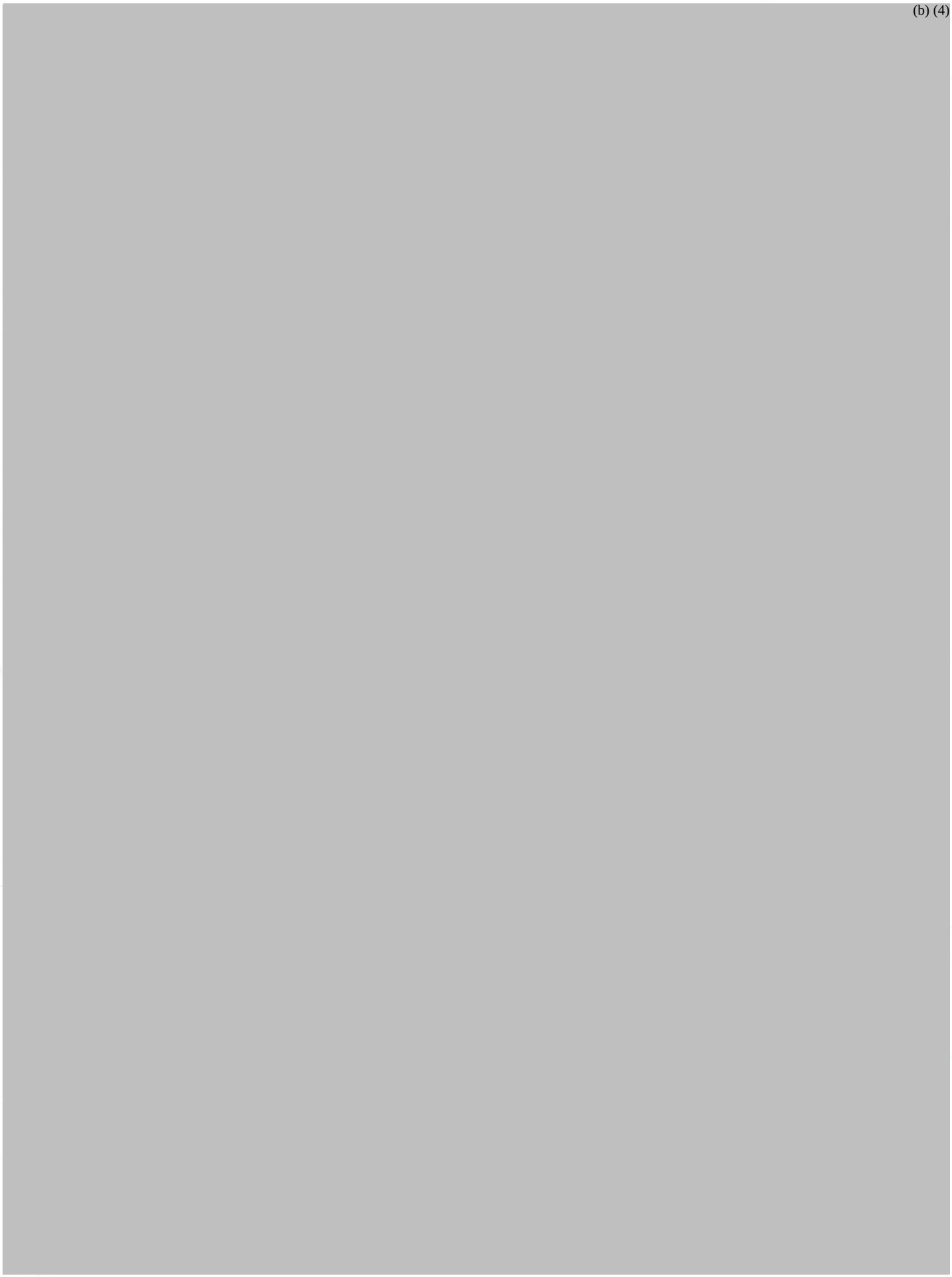
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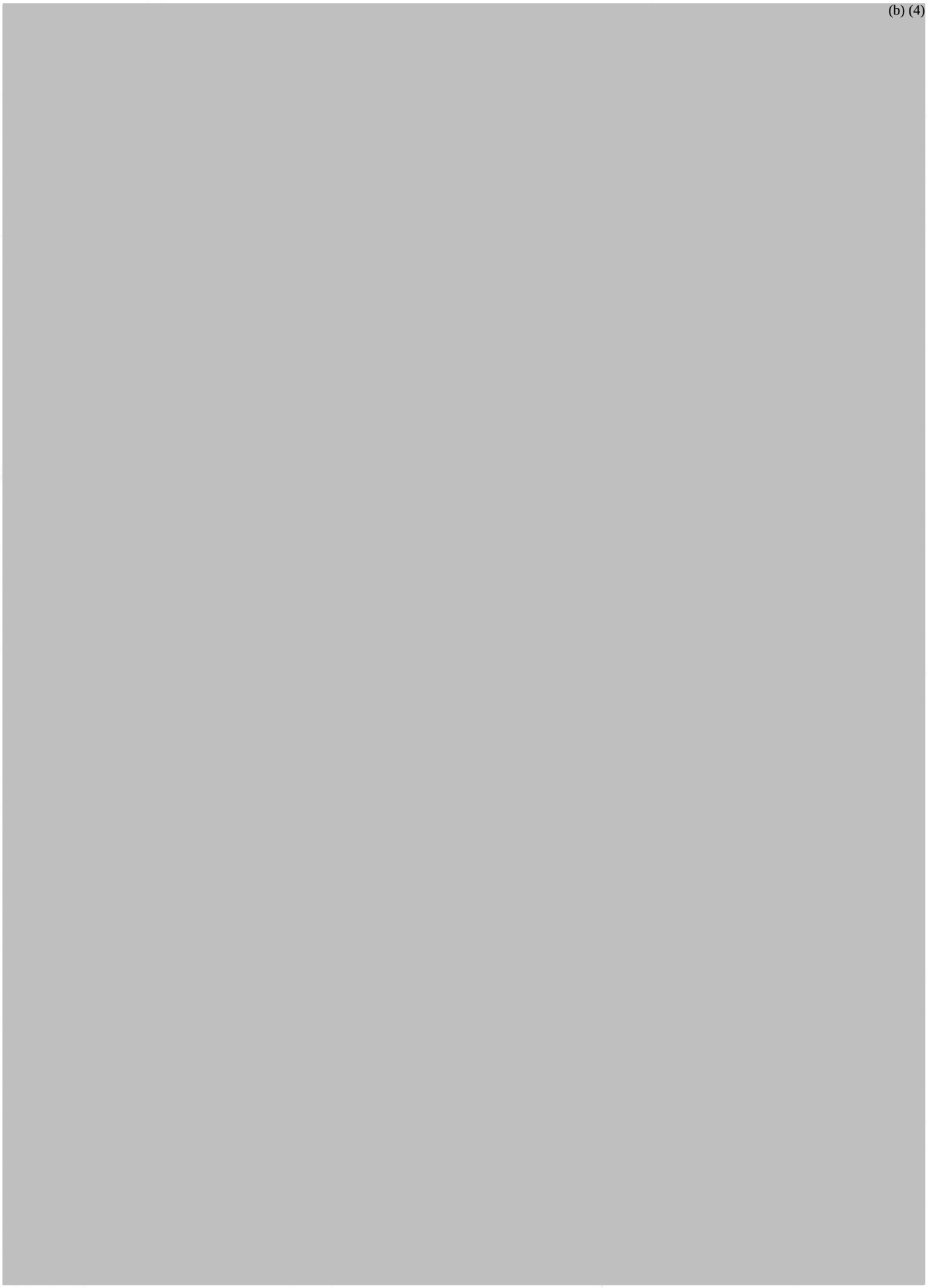

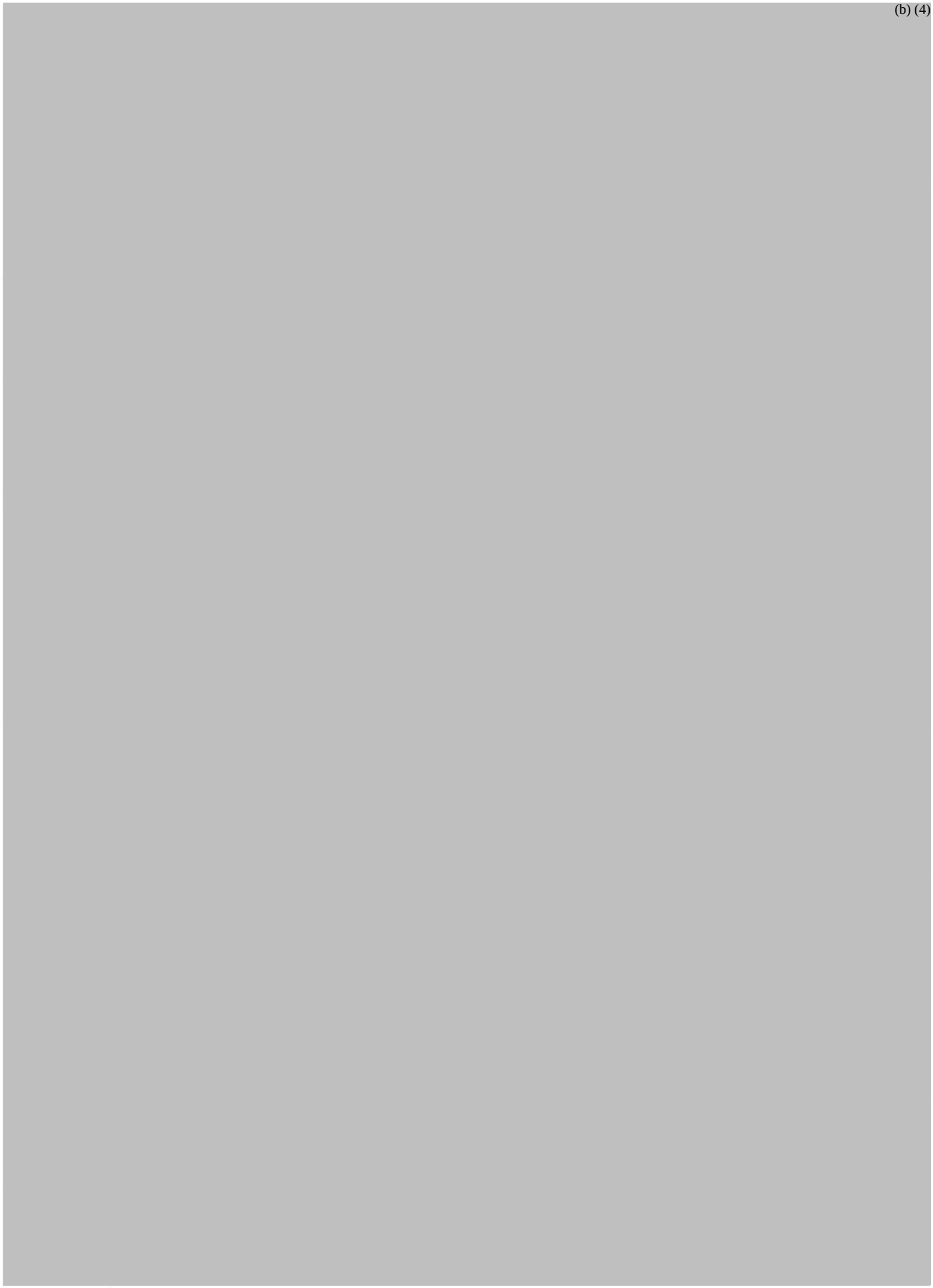


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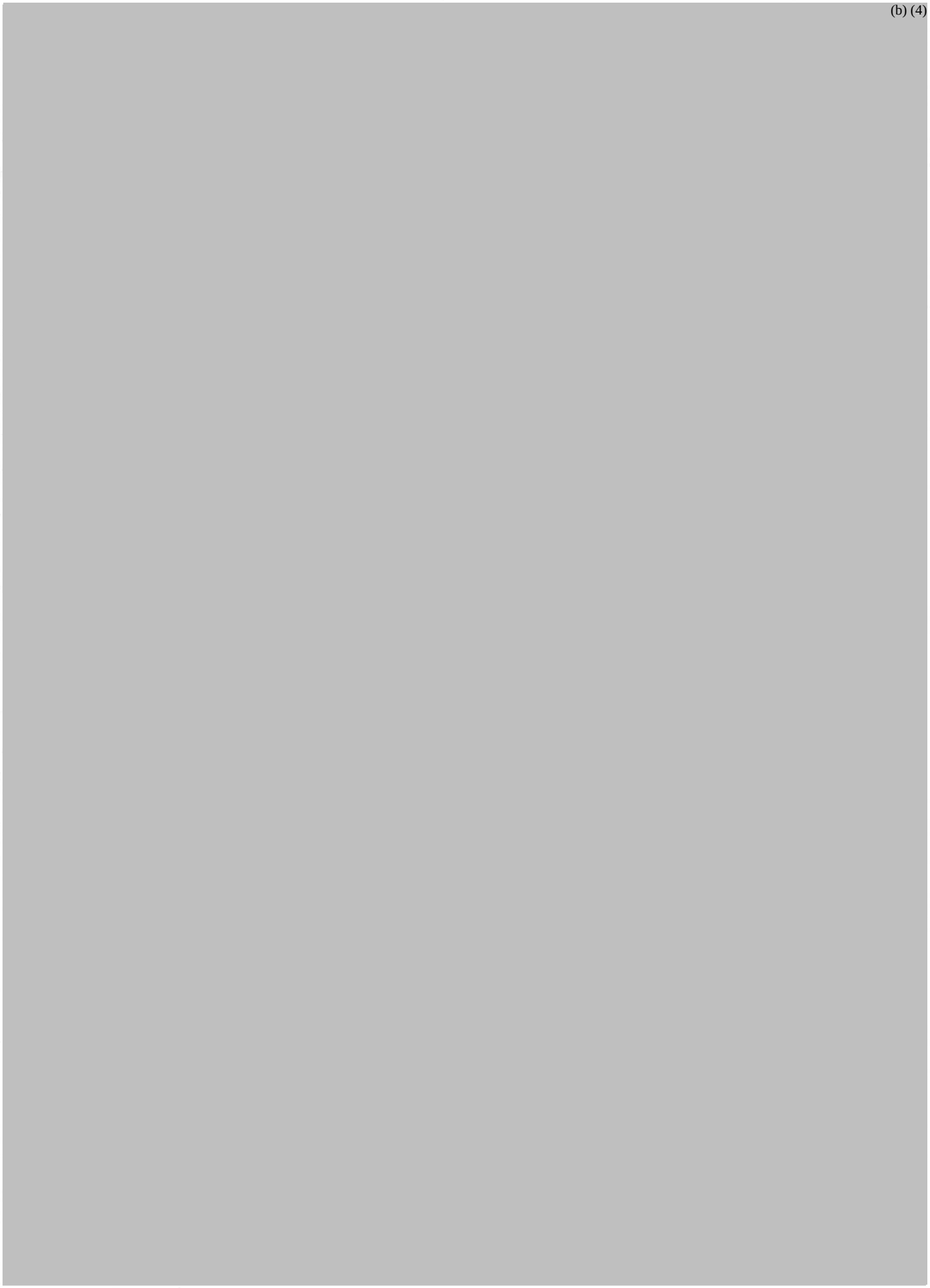
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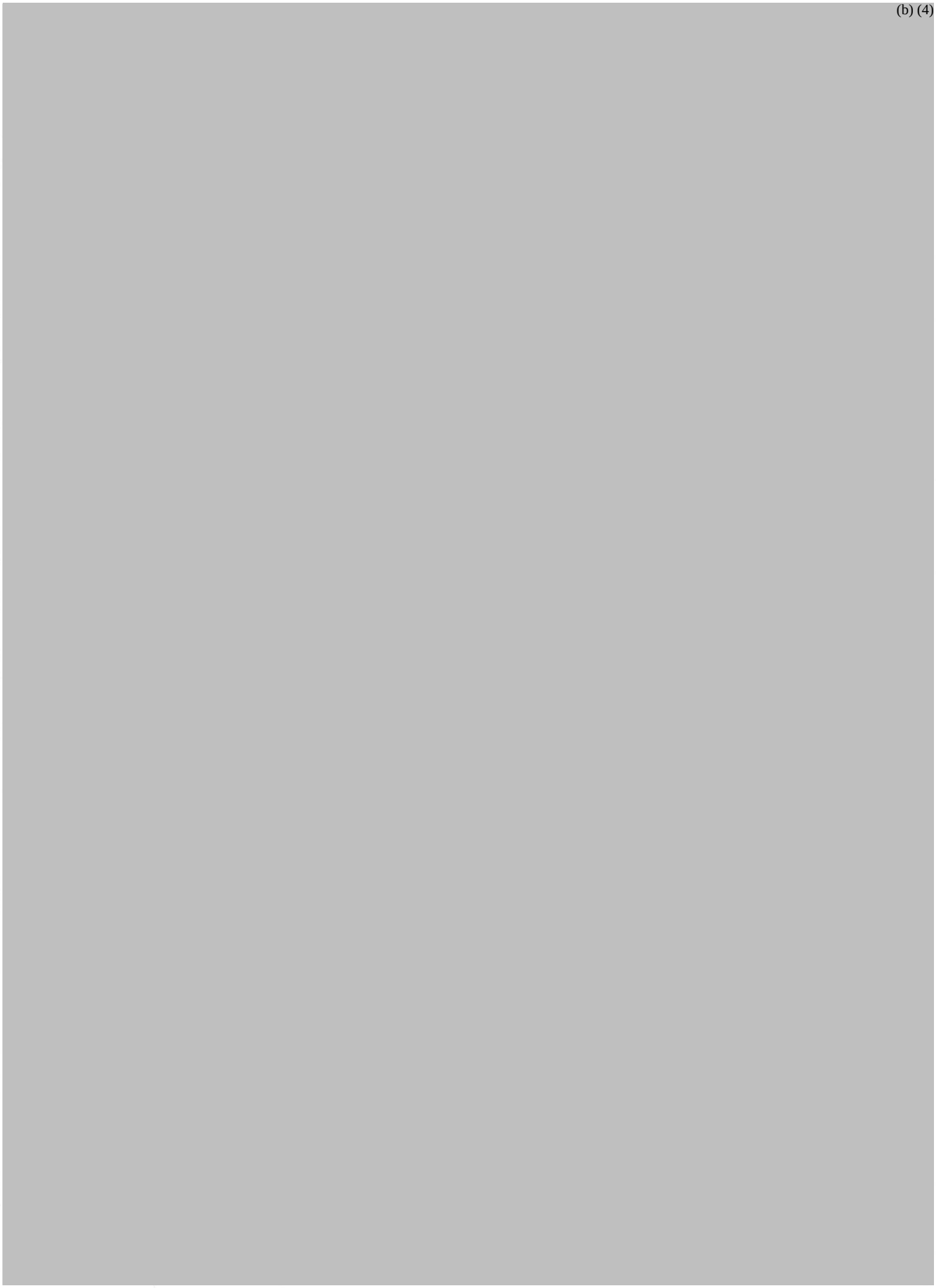
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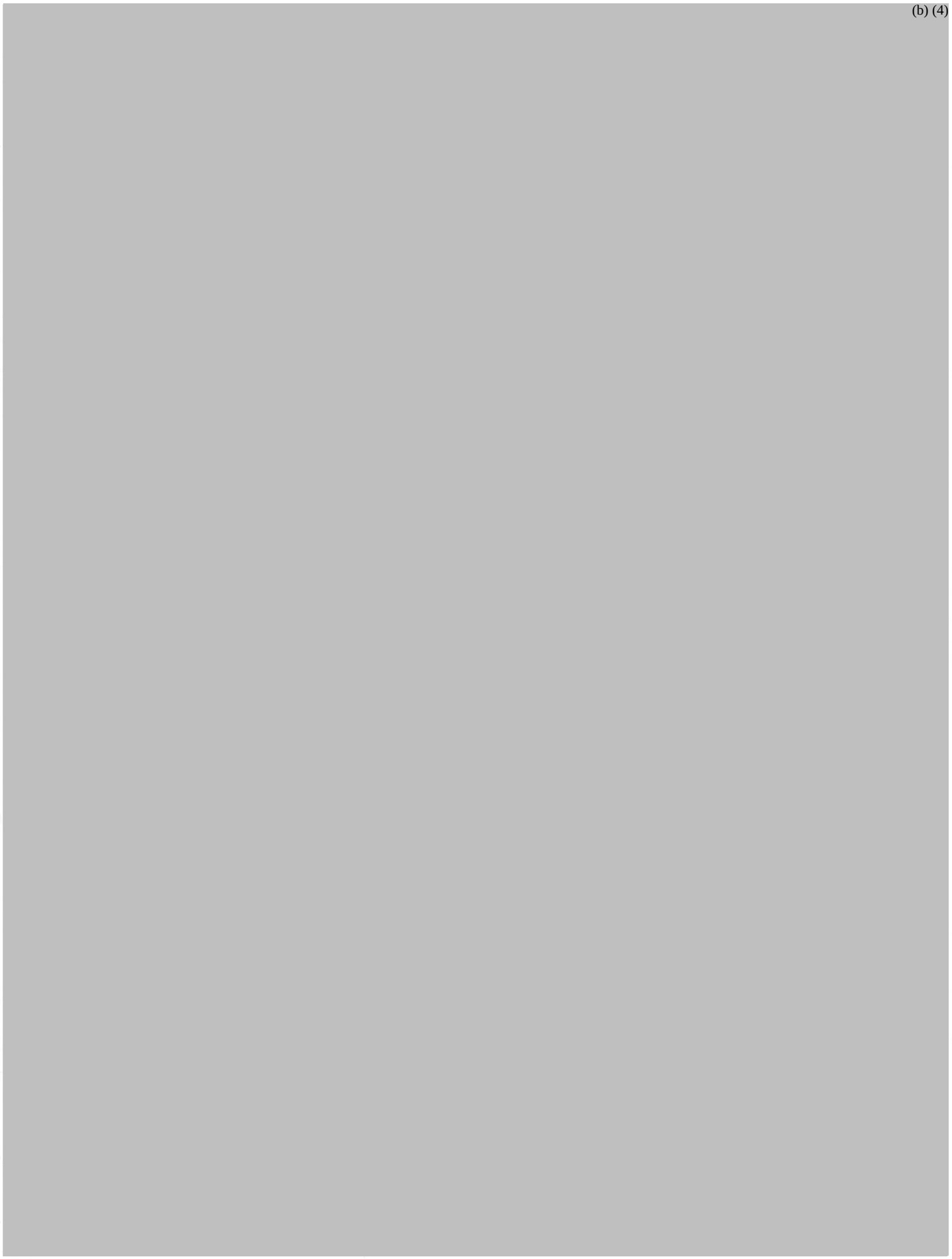
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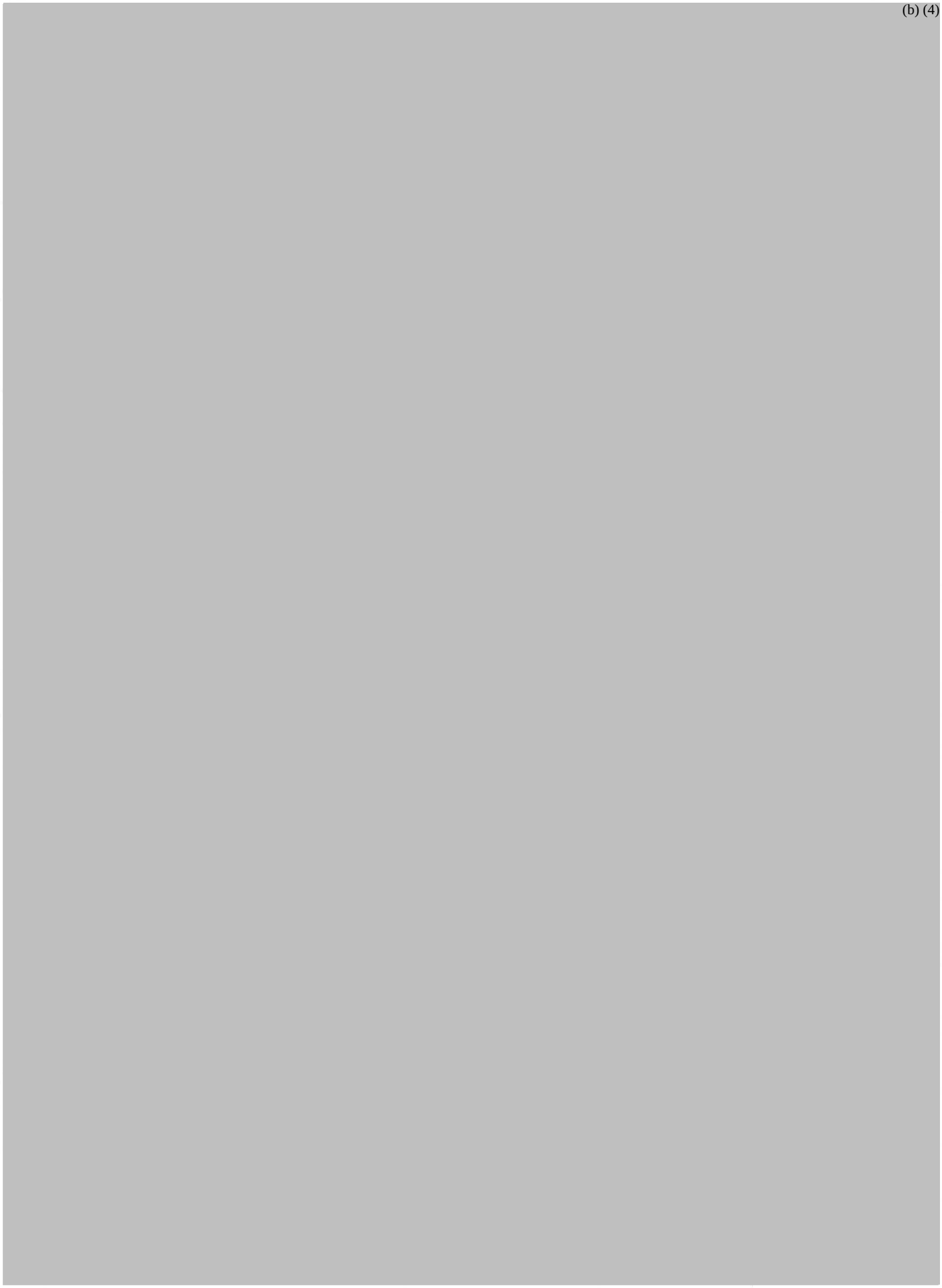
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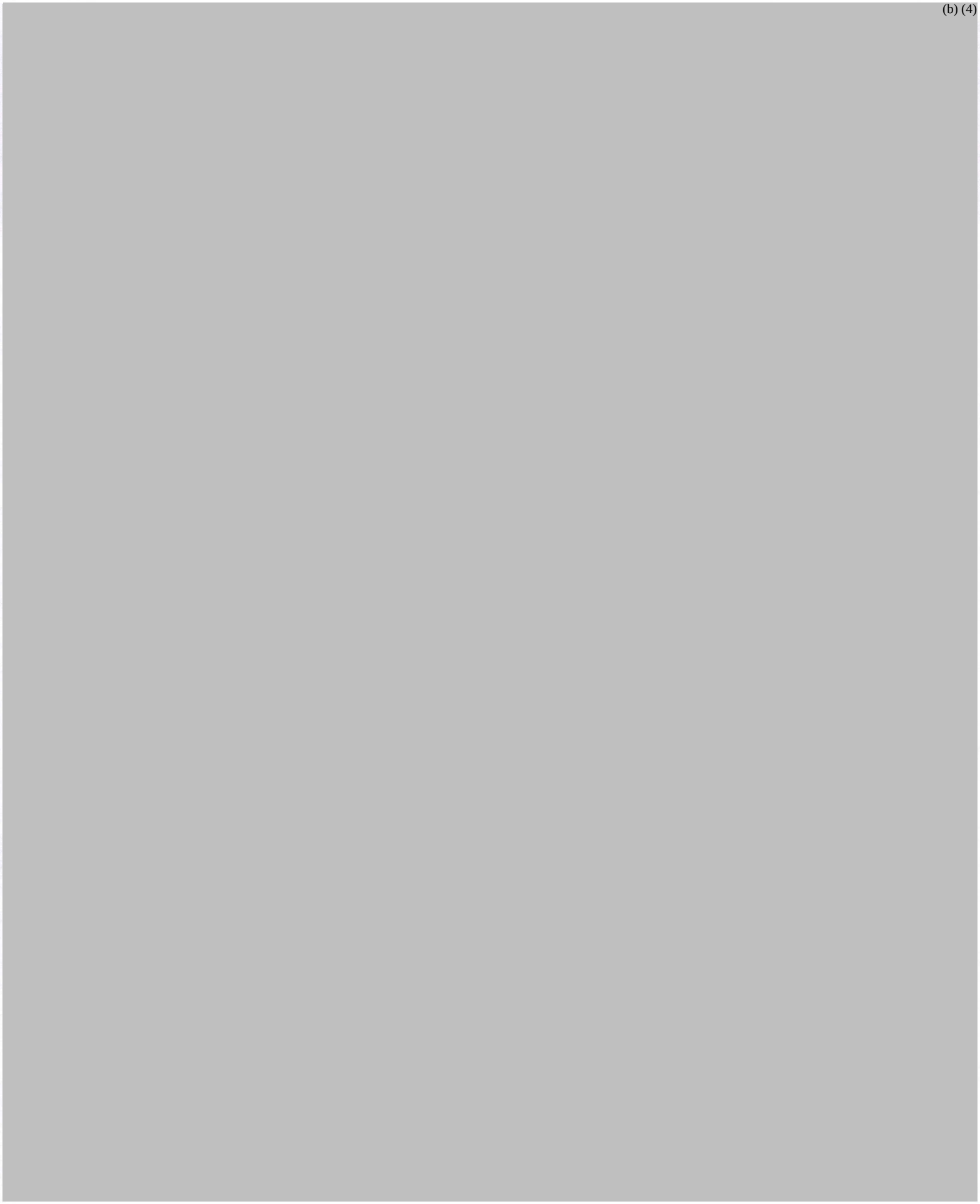
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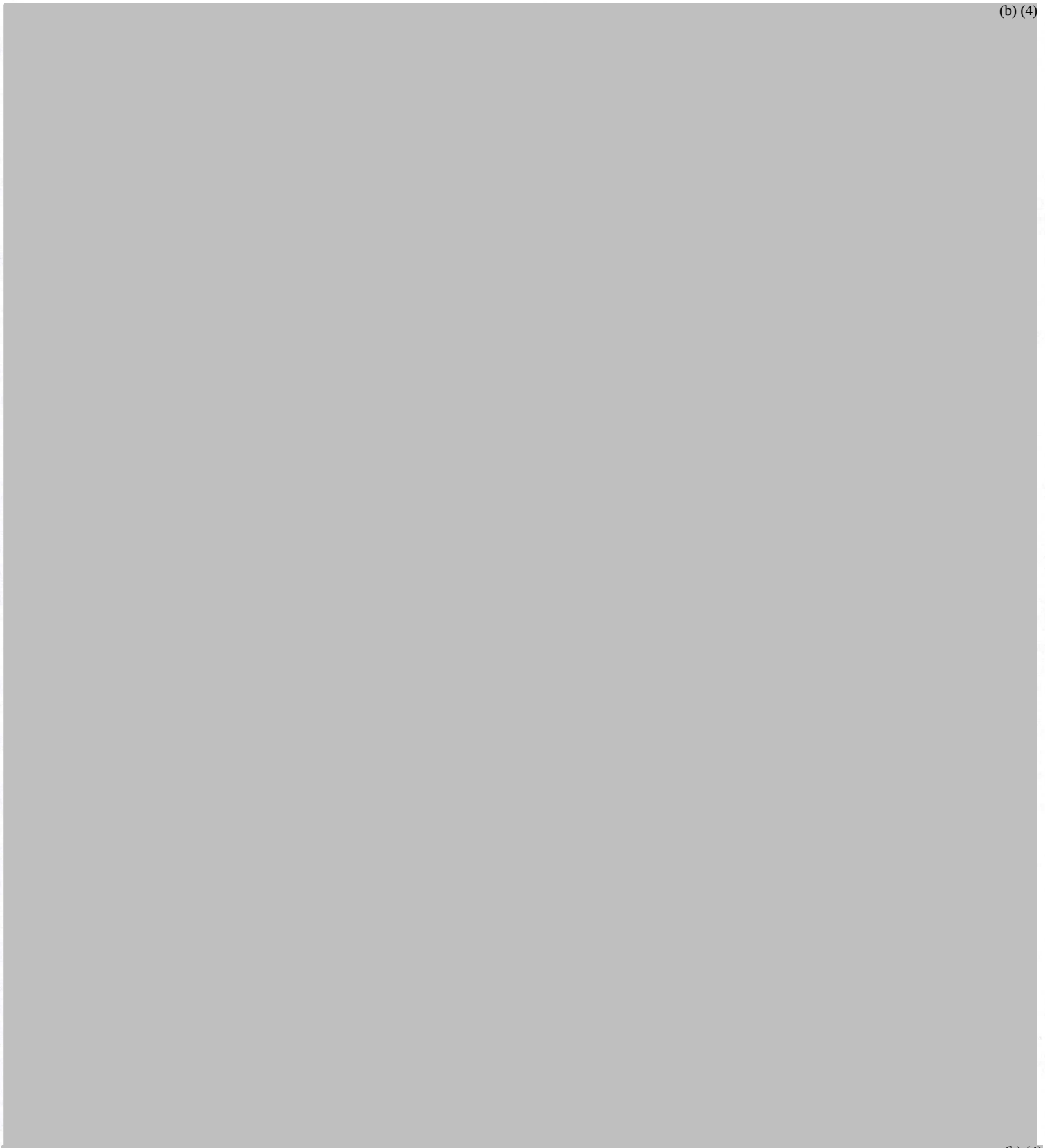
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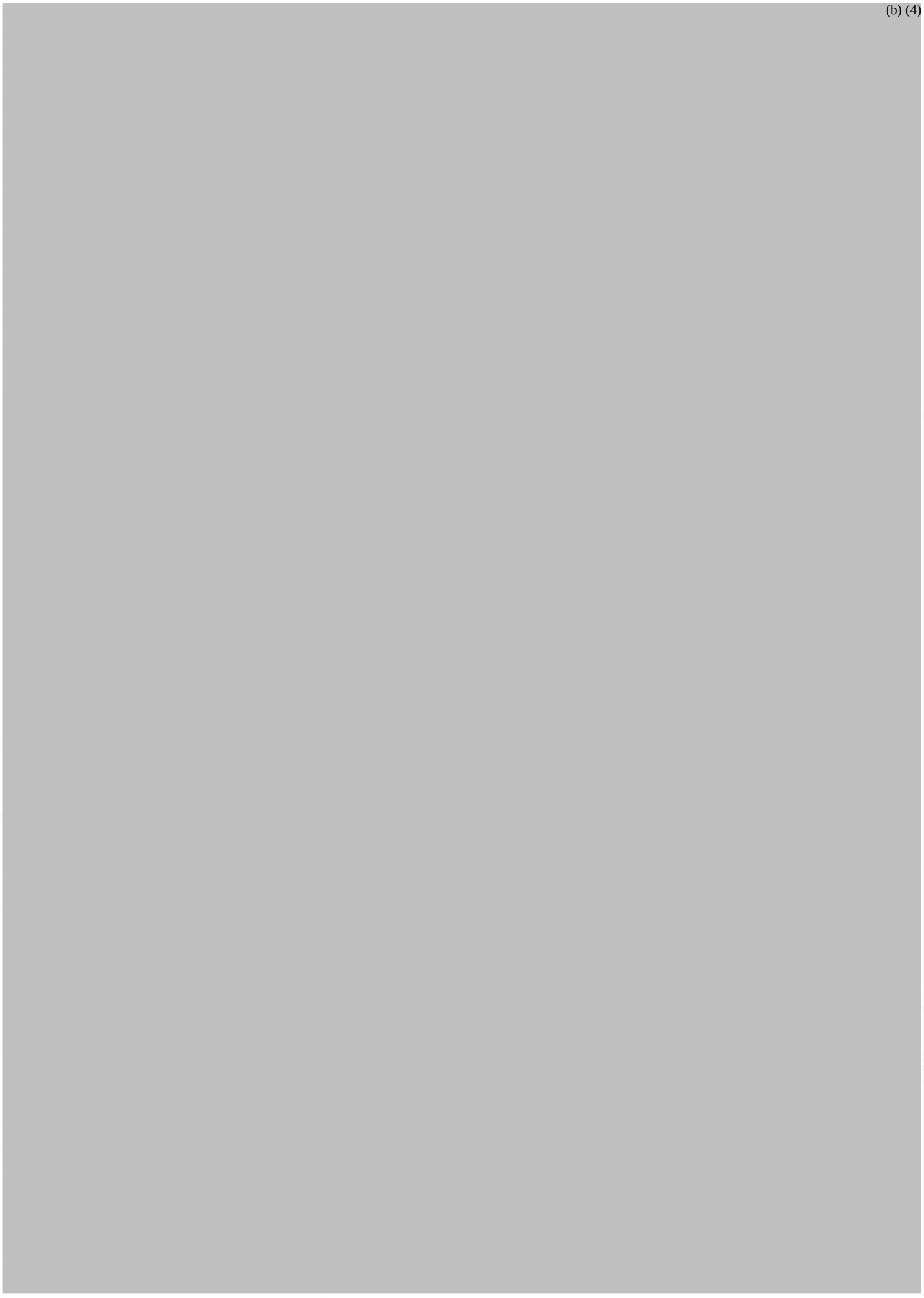

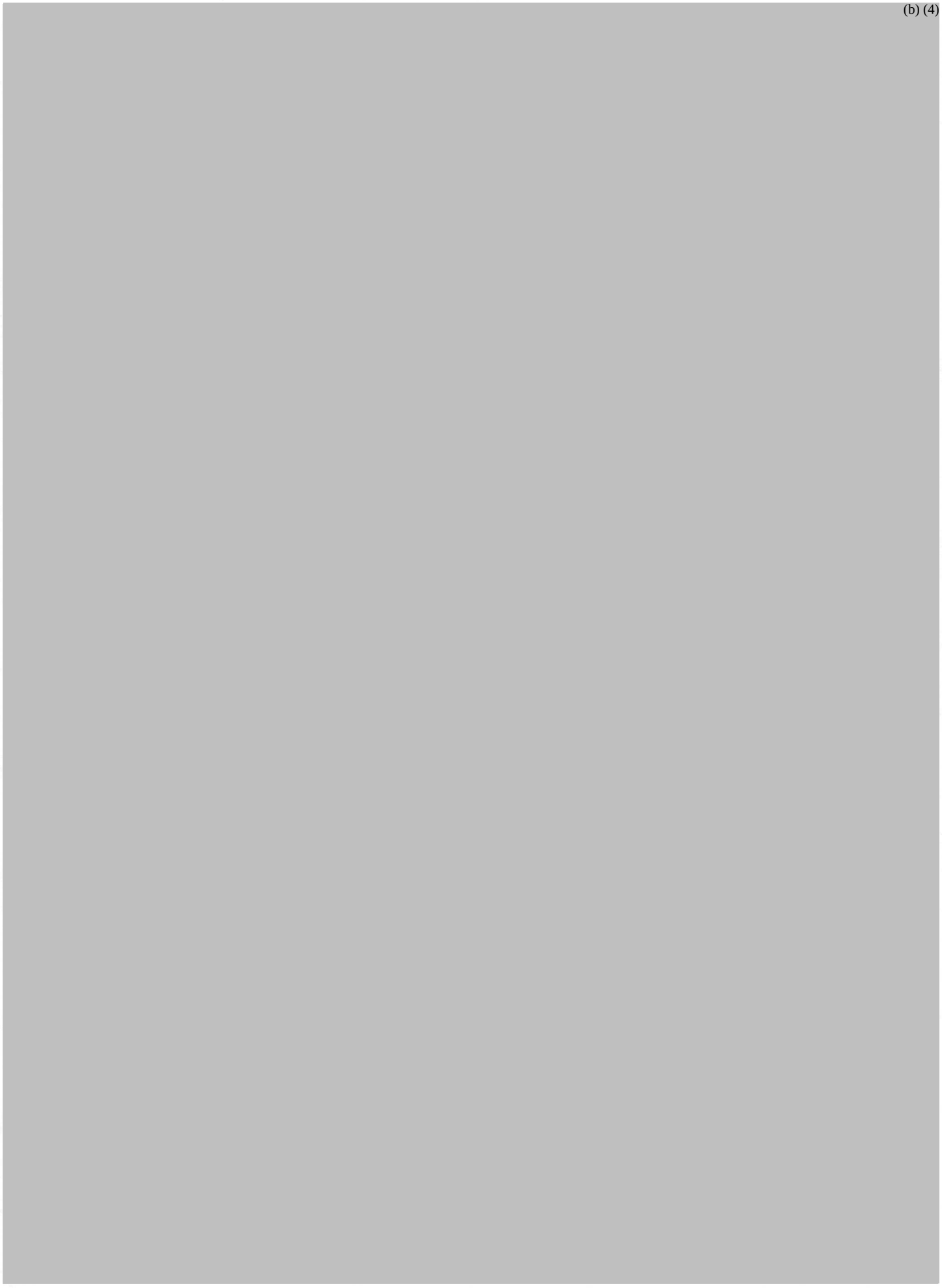

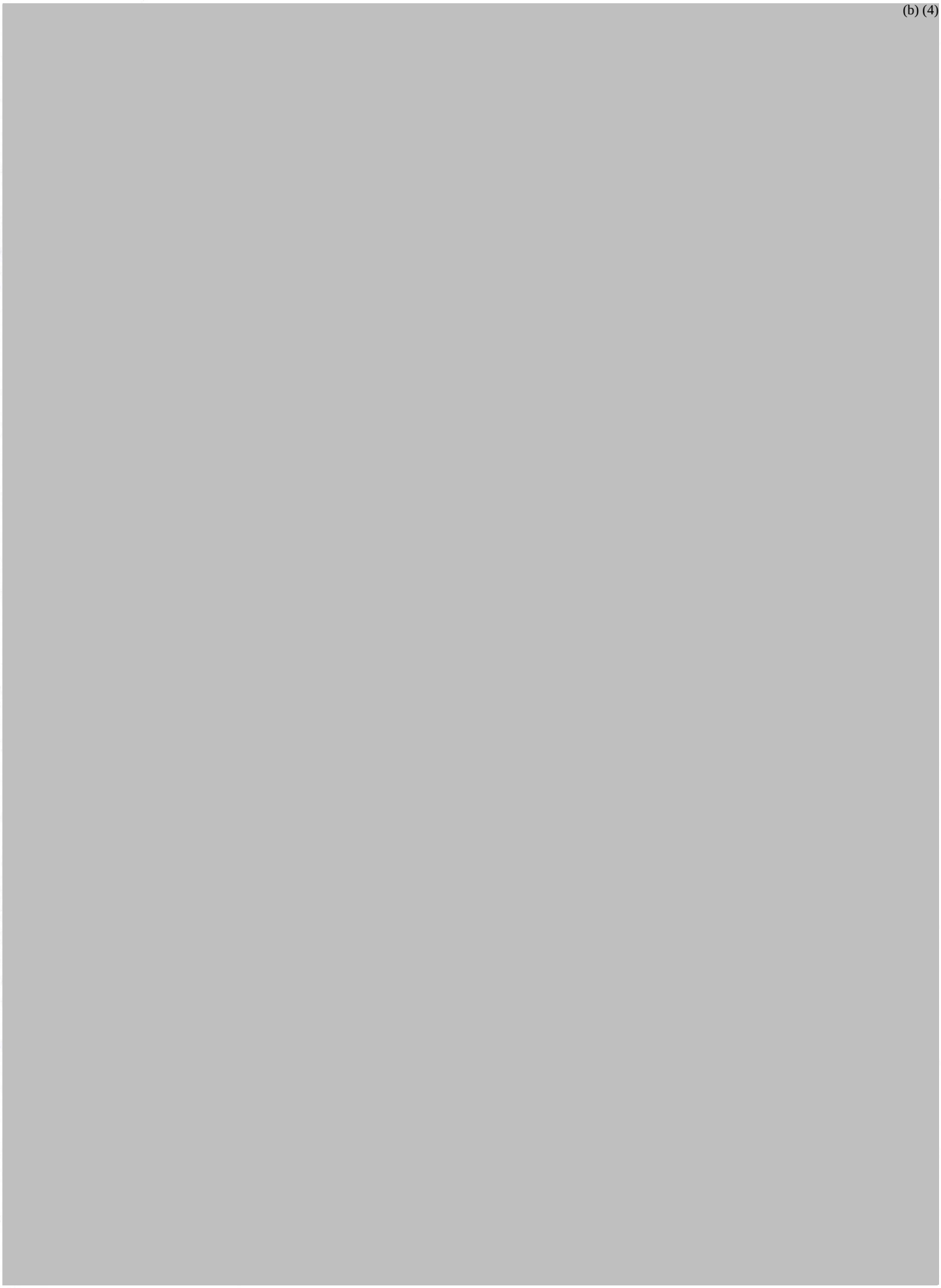

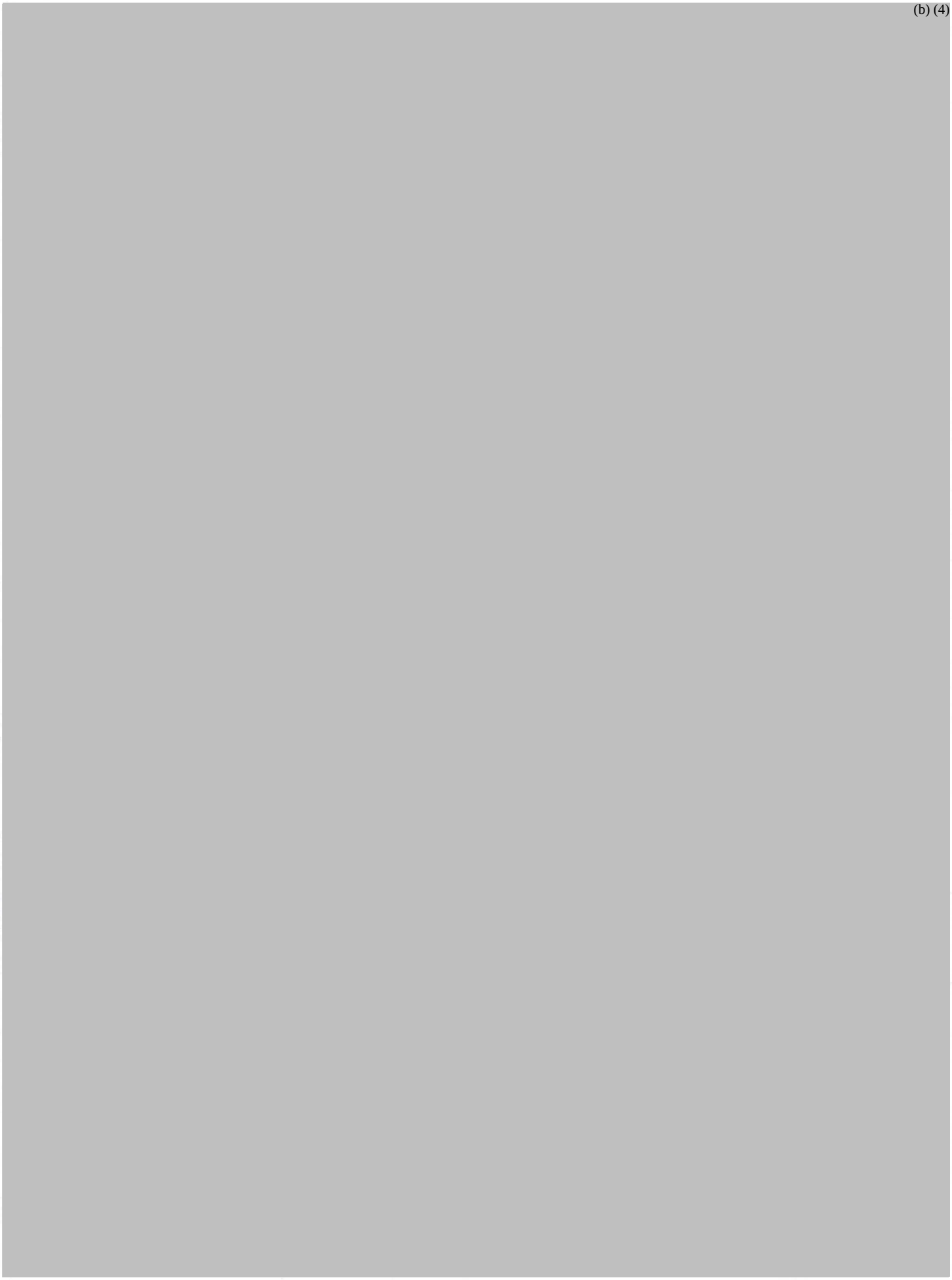
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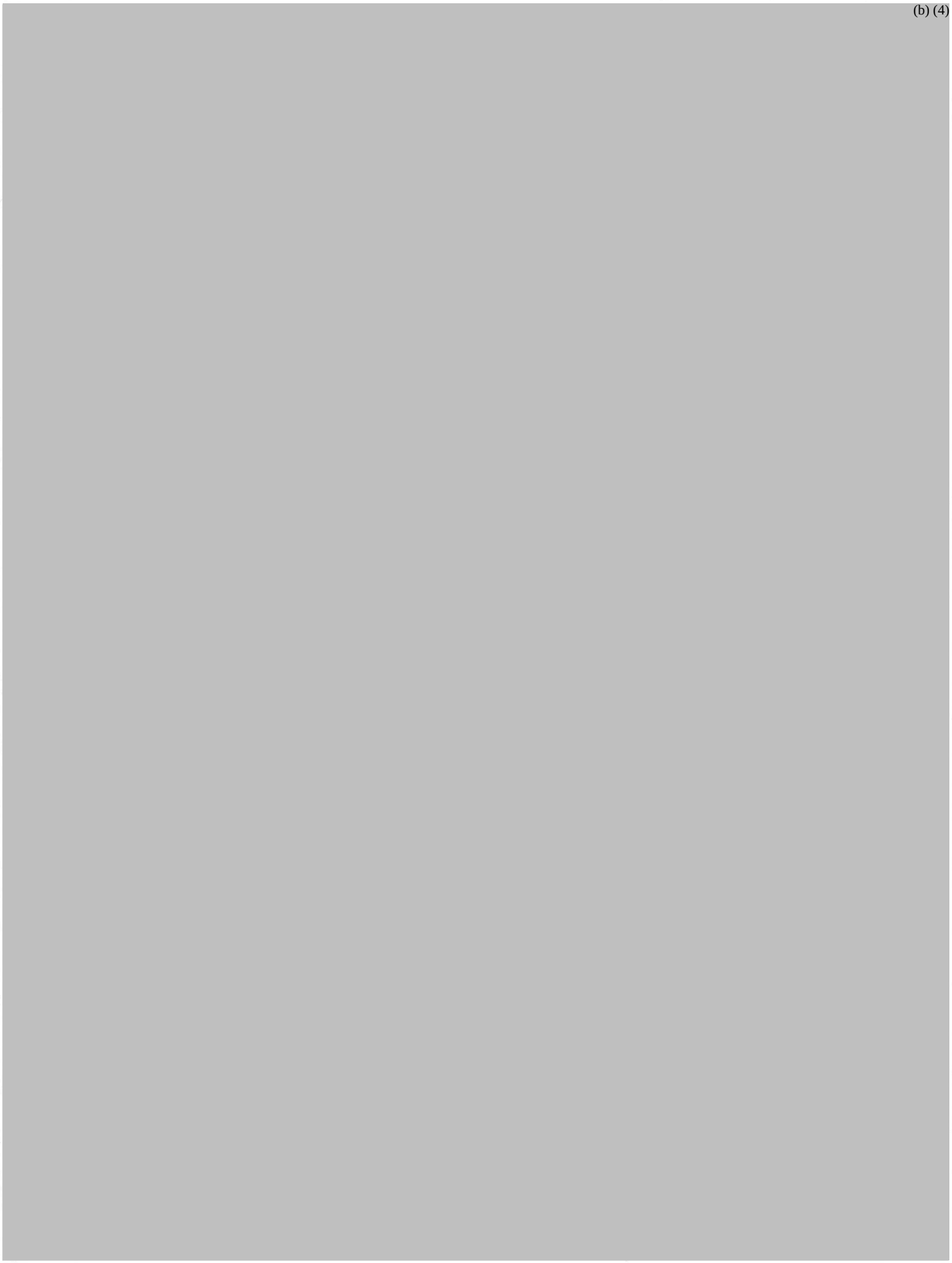
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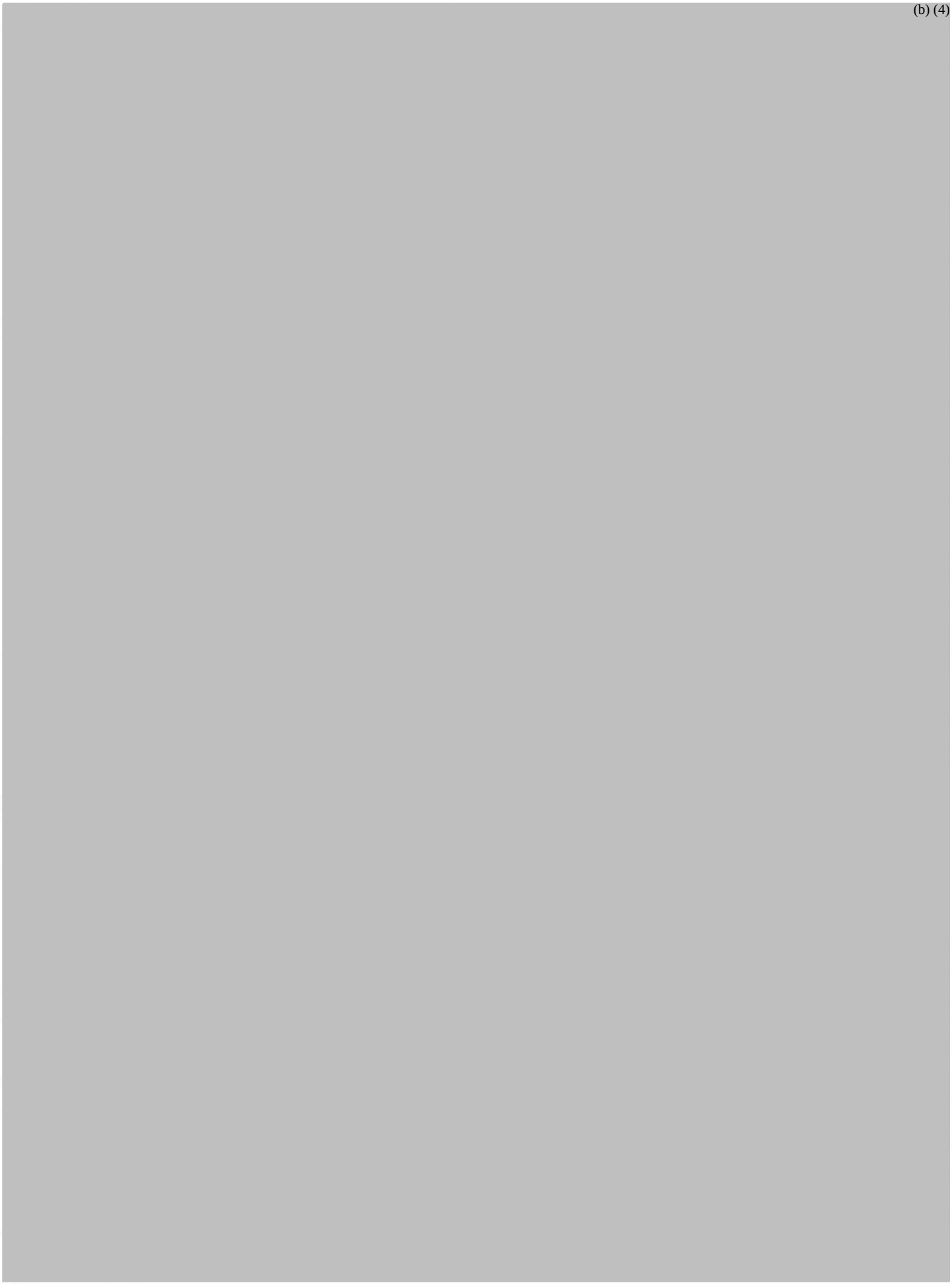

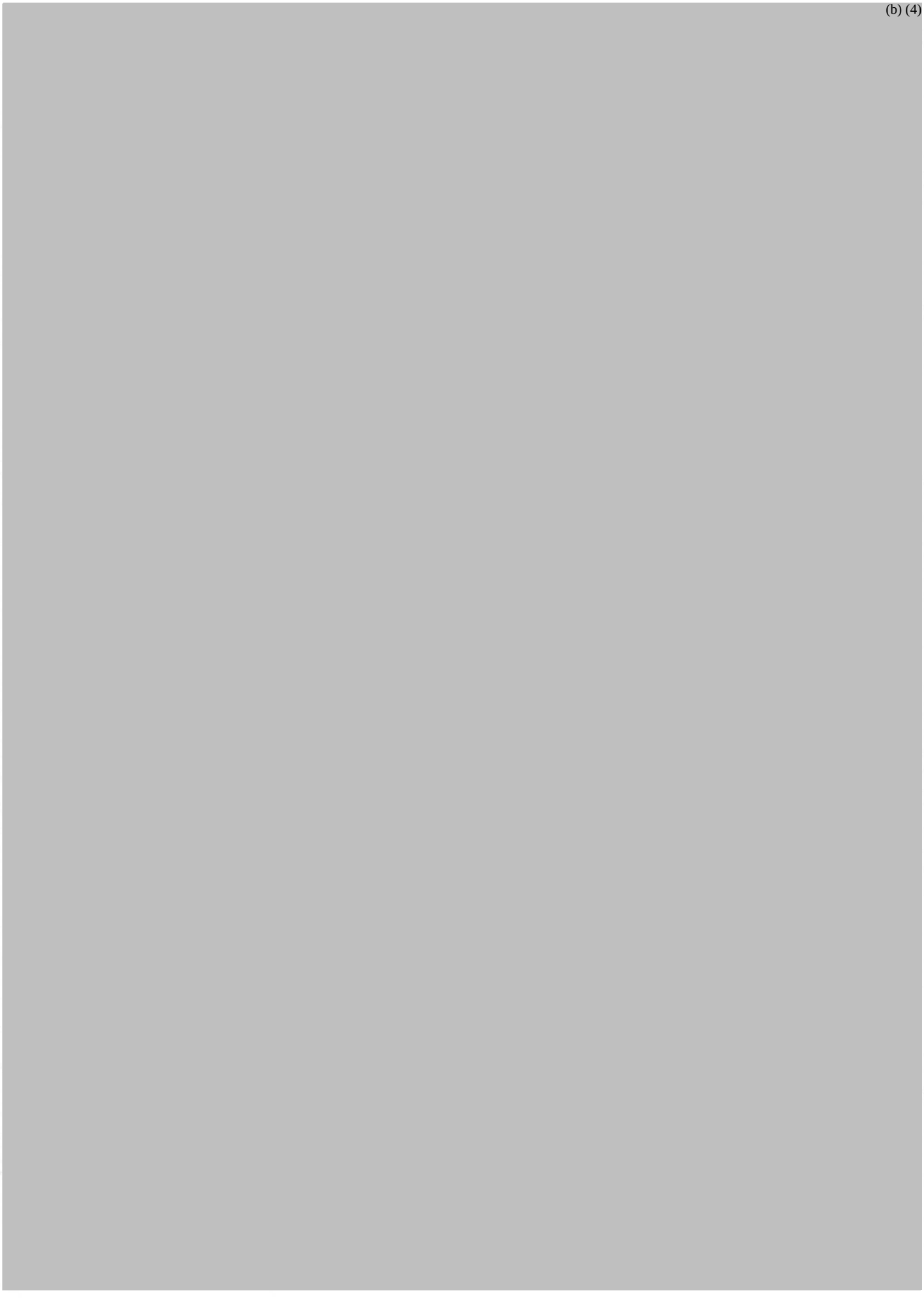

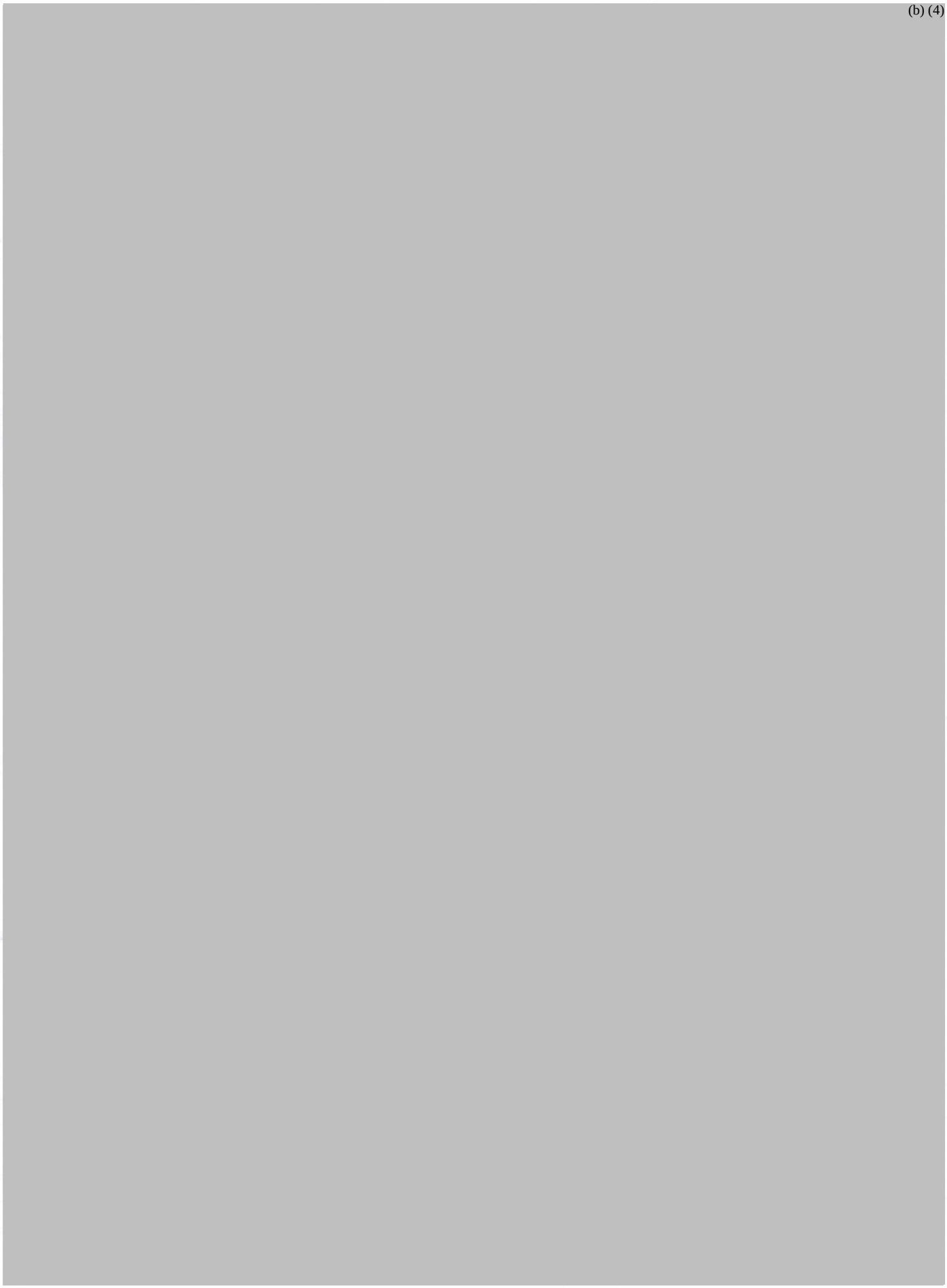

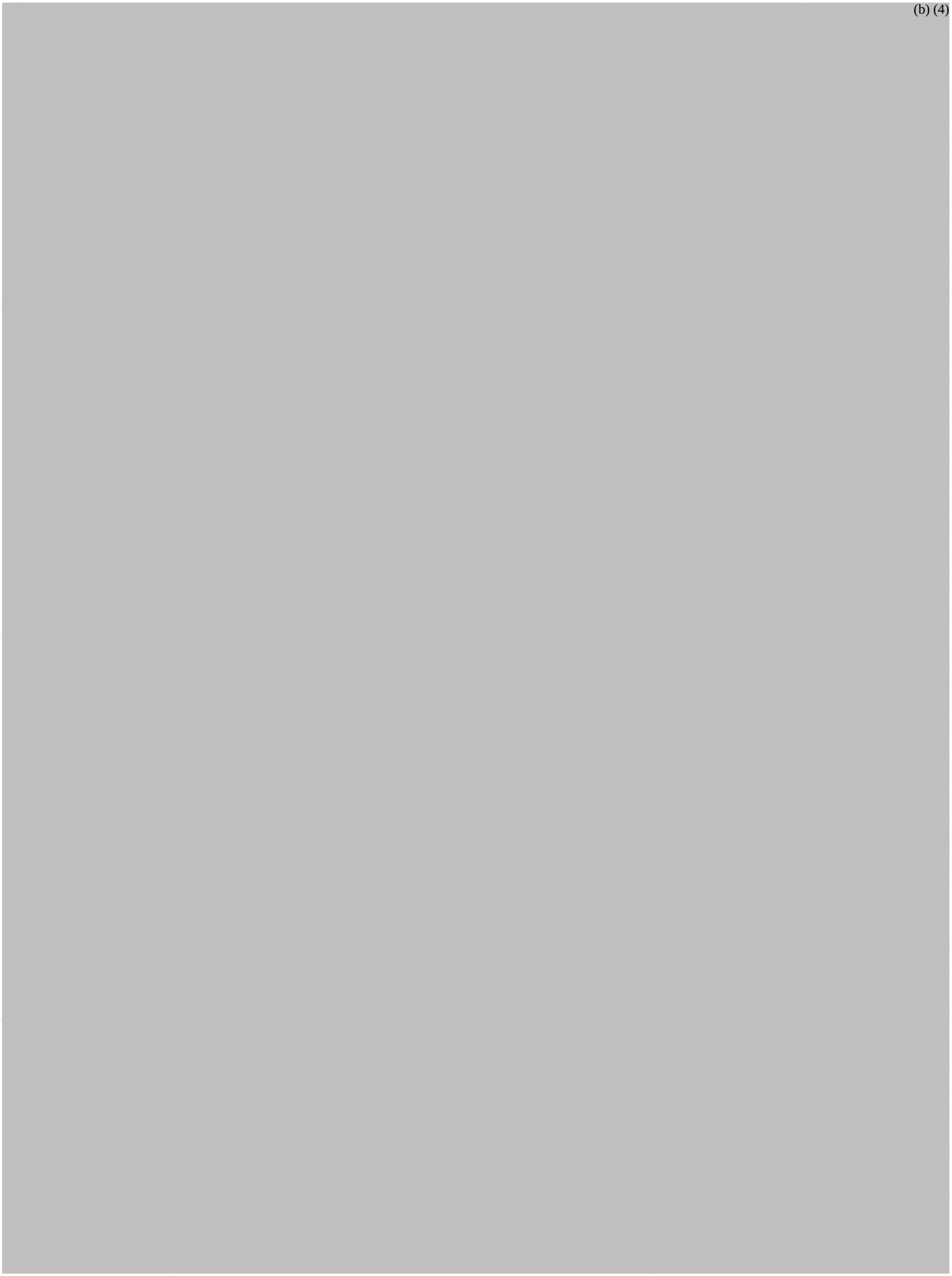
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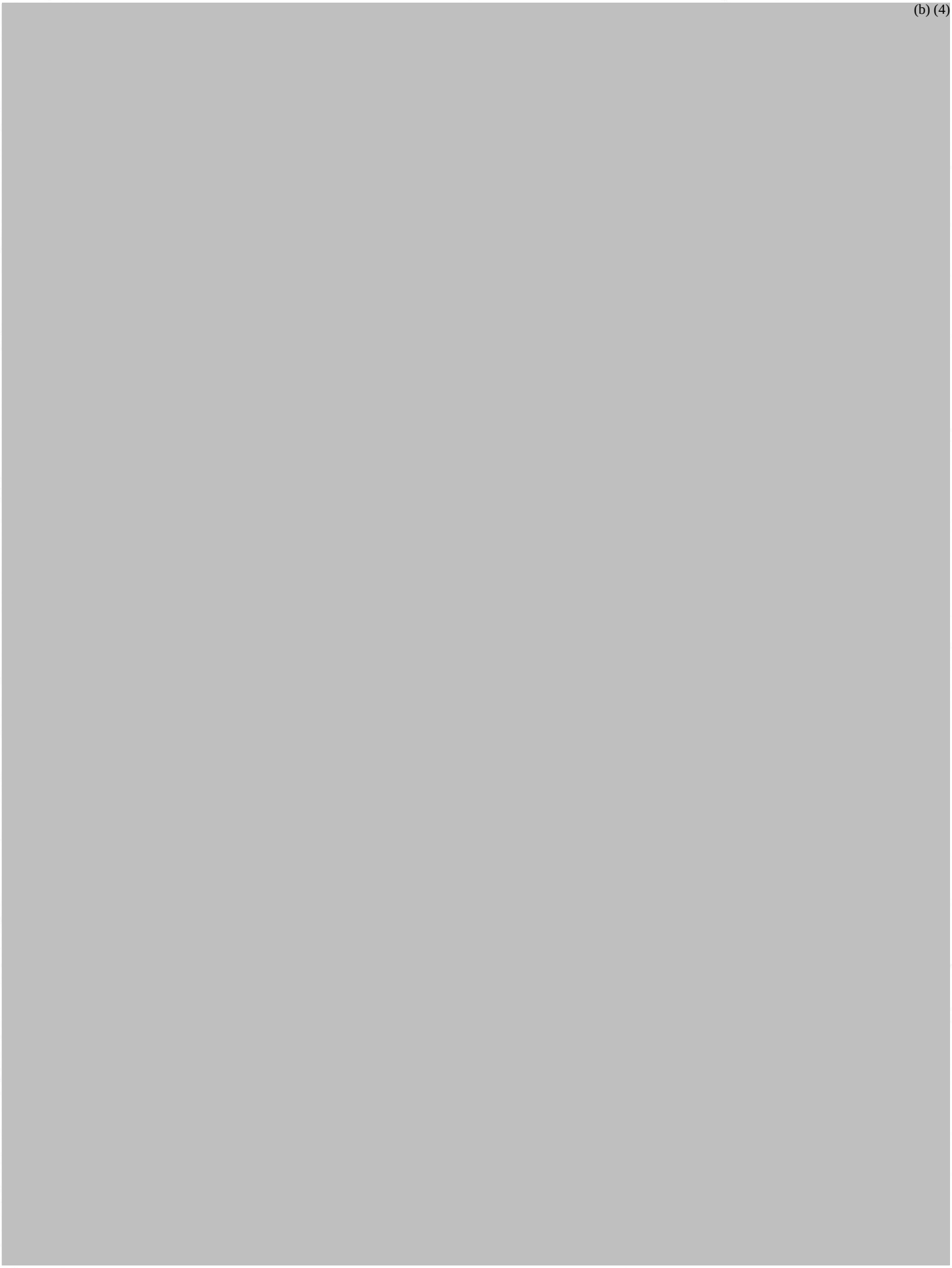

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