Journal of Helminthology (2014) **88**, 511–514
doi:10.1017/S0022149X13000394
© *Cambridge University Press 2013*

The use of soy protein polymers as a release device for nematophagous fungi in the control of parasitic nematodes in ruminants

M.F. Sagüés[1,2]*, P. Purslow[3], A.S. Fernández[4], L.E. Iglesias[2], L.A. Fusé[2] and C.A. Saumell[2]

[1]Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires B7000GHG, Argentina: [2]Área de Parasitología y Enfermedades Parasitarias, Departamento de Sanidad Animal y Medicina Preventiva, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina: [3]Department of Food Science, Ontario Agricultural College, University of Guelph, Guelph, Canada: [4]BioNem Research Centre, Guelph, Canada

(Received 23 October 2012; Accepted 18 April 2013; First Published Online 11 June 2013)

Abstract

This trial was conducted to evaluate the predatory activity of *Duddingtonia flagrans* incorporated into soy protein-based polymers as a controlled-release device (CRD). The rate of fungal release from the polymers and time of residence of the CRD in the rumen of a cannulated sheep was also determined. After administration to the sheep, the CRD was extracted at weekly intervals over a month for observation of its physical structure and faeces were collected to observe the subsequent predatory activity of the fungus in Petri dishes with water-agar 2% and *Panagrellus* spp. as bait. The CRD slowly degraded in the rumen over 4 weeks and liberated *D. flagrans* into the faeces. The formulation of the soy protein-based polymers did not affect the predatory activity of the fungus. The study demonstrates that biodegradable soy protein polymers could potentially improve the use of nematophagous fungi for controlling nematode parasites of ruminants.

Introduction

The potential advantages of using nematophagous fungi as a biological control tool to reduce pasture infectivity without residues in animals and the environment are recognized worldwide. If biological control is to become a real alternative in the fight against gastrointestinal nematodes then development of an effective means of delivery of nematophagous fungi is a challenge that must be overcome. Several forms have been evaluated previously for the administration of the fungus, including mineral blocks (Waller *et al.*, 2001b), energy blocks (Sagüés *et al.*, 2011), supplementation with fungus-containing grains (Waller *et al.*, 2001b), multi-nutritional pellets (Casillas Aguilar *et al.*, 2008) and fungus-containing alginate pellets (Araújo *et al.*, 2000; Ribeiro Braga *et al.*, 2009). Each of these modes of administration could be potentially useful depending on which livestock production system they would be applied to. However, since they generally depend on continuous administration of fungal spores to maintain effective fungal levels in faeces, they do not seem suitable in the case of extensive livestock production.

Another strategy is to administer fungal spores in a form or device which releases them slowly and over an extended period, i.e. a controlled-release formulation or controlled-release device (CRD). Waller *et al.* (2001a) tested an intraruminal prototype CRD and found that *Duddingtonia flagrans* chlamydospores were released constantly up to 23 days after being implanted in the

*Fax: + 54 249 4439850
E-mail: federica@vet.unicen.edu.ar

rumen of fistulated sheep. Several veterinary drug delivery systems currently exist, but are manufactured from non-degradable polymers or metals. Since biodegradable CRDs do not have to be removed from the animal, the study of biopolymer-based CRD formulations has increased in recent years (Winzenburg *et al.*, 2004).

Soy protein polymers (SPP) have been proposed as vehicles for the controlled release of drugs, micronutrients and biopesticides (Rosemberg & Young, 1993; Vaz *et al.*, 2003; Maltais *et al.*, 2010). Soy protein isolate is biodegradable, environmentally friendly and readily available from an abundant renewable resource. It has been widely used as a functional ingredient in many different food products (Maltais *et al.*, 2005) and has also been considered as an interesting starting material for the development of new materials and devices for biotechnological and biomedical utilization (Caillard *et al.*, 2009; Maltais *et al.*, 2009, 2010; Song & Zhang, 2009; Song *et al.*, 2009). Sagüés *et al.* (2012) evaluated the release of chlamydospores of *D. flagrans* through films made from soy protein in distilled water and ruminal fluid, with encouraging results. CRDs from soy protein polymers designed to release *D. flagrans* chlamydospores over extended periods therefore appear to be an attractive prospect for future development. The aim of the present study was to evaluate *in vivo* a soy protein-based CRD for the release of *D. flagrans* chlamydospores in sheep.

Materials and methods

Fungal material and viability of chlamydospores

The trial was conducted on the Experimental Farm, Faculty of Veterinary Sciences, Tandil, Argentina (37°17′34″S, 59°5′W). The local isolate 03/99 of *D. flagrans*, previously obtained from the same site (C. Saumell, pers. comm.), was used for the study. The fungus was cultured in Petri dishes with pure water-agar (2%) at 24°C for 4 weeks. Chlamydospores were gently rinsed off from the Petri dishes with sterile water and counted using a Neubauer haemocytometer to estimate their numbers until a concentration of 8×10^8 chlamydospores in 20 ml of distilled water was reached. This concentration is deliberately higher than that used in previous trials (Githigia *et al.*, 1997; Larsen *et al.*, 1998).

The proposed formulation of the soy protein polymers included dialdehyde starch as a protein cross-linking agent. Prior to the CRD preparation, it was necessary to evaluate the survival of fungal spores when brought into contact with dialdehyde starch. The chlamydospores were incubated in three different concentrations of dialdehyde starch (0.24, 1.0 and 5.0 wt%) in 2% agar in water on Petri dishes. Four replicates for each concentration were prepared. *Panagrellus* spp. was added in order to promote fungal germination and growth. The dishes were incubated at 25°C for 1 week and inspected daily by optical microscopy to detect the presence of three-dimensional fungal networks.

Controlled release device

The preparation of the CRD was carried out at room temperature and followed the technique described by Sagüés (2012). Sixty grams of powdered soy protein isolate SPI (Archers Daniels Midland Co., Decatur, Illinois, USA) were mixed with 60 g of 1N $Ca(OH)_2$ (Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) in a kitchen blender. Ten grams of glycerol (Sigma-Aldrich Co.) were added, the mixture was re-mixed and 3 g of dialdehyde starch (Sigma-Aldrich Co.) were finally added. Two hundred grams of distilled water and 20 ml of distilled water containing 8×10^8 chlamydospores of *D. flagrans* were then mixed in and 75 g of Ceria stabilized zirconium oxide beads (Fox Industries Inc, Baltimore, Maryland, USA) were added to increase the density. This material was extruded repeatedly through a sausage stuffer, to promote the loss of air, and put into a natural sheep gut casing that had been previously hydrated in distilled water at 40°C. Portions of 4 cm in length were separated and dried for 20 h at 30°C.

In-vivo *placement and retrieval of the controlled release device*

A 4-year-old male Corriedale sheep was kept in a roofed pen with cement floor and fed bales of alfalfa and a commercial concentrate for sheep, both free of nematophagous fungi. Once dry, the cylindrical devices were placed inside a bag made out of fine-meshed nylon mosquito net, which was then placed into the rumen of the cannulated sheep. The cannulation surgery was carried out following the Animal Procedures and Management Protocols approved by the Ethics Committee according to the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Sciences, UNCPBA (http://www.vet.unicen.edu.ar/html/Comite%20Bienestar%20Animal/ComiteBienestarAnimalActas.html)

A nylon thread was used to fix the bag externally to the cannula in order to retrieve it weekly for examination. The mosquito net allowed the flow of the ruminal liquid into the bag as well as the close contact between the CRD and ruminal content. The device was retrieved weekly for macroscopic evaluation over four consecutive weeks. The procedure described above was repeated three times using the same ruminally cannulated sheep.

Faeces were collected daily from day 0 until day 28 post-administration of the CRD to assess the presence and predatory capacity of the fungus. Up to 2 g of faeces were weighed and placed in each of four Petri dishes containing 2% water-agar, incubated at 25°C and observed daily. *Panagrellus* spp. was added in order to stimulate the formation of three-dimensional networks and nematode capture. Petri dishes were checked daily over 28 days for evidence of the presence or absence of three-dimensional networks.

Results and discussion

Fungal spores added to the Petri dishes containing dialdehyde starch at different concentrations in 2% water-agar and nematodes remained viable. The predatory activity at the concentration of 0.24% dialdehyde starch was 75% and at the concentrations 1% and 5% dialdehyde starch it was 100%. The chlamydospore viability was confirmed as they germinated and the fungus formed its three-dimensional networks stimulated by the presence of

free-living nematodes. These initial trials indicated that formation of the CRD using dialdehyde starch as a cross-linking agent would not interfere with chlamydospore viability. As early as the second day after administration of the CRD to the cannulated sheep, the formation of three-dimensional fungal networks and capture of nematodes were observed in faecal samples. The CRD was retrieved weekly to conduct a visual check of its physical structure. As intended, the device slowly shrank in size as it was degraded by digestive enzymes, but remained intact in the rumen over the 4-week duration of the experiment.

This study provides strong evidence that a CRD based on soy protein polymers is a practical possibility that could be used in parasitic nematode control of ruminants. Not only did the chlamydospores withstand the very moderate pressures required to formulate the soy-based polymers into a cylindrical device, but, furthermore, they were detected in faeces 2 days after administration of the prototype CRD. At least 10^6 chlamydospores must be liberated from such devices into the rumen in order to substantially reduce the number of nematode infective larvae that succeed in developing in the faeces of parasitized sheep (Waller *et al.*, 2001a). The main issue that now needs to be addressed is the formulation of a CRD that continuously and evenly releases approximately 10^6 chlamydospores per day over the lifetime of the device. This could achieve a profound reduction in the number of infective larvae on pasture (Larsen *et al.*, 1998). Ruminal movements play a major role in the digestion process; the length of forage consumed by ruminants is reduced by the initial chewing of particles to 1–2 cm or less. The majority of particles moving after digestion through the reticulo-omasal orifice are 2–3 mm long (Cunningham, 1996). An intraruminal CRD must resist this breakdown process and remain intact so as to fulfil its function of slowly releasing chlamydospores in faeces for at least 1 month. The material used to manufacture the CRD must hence be resistant to mechanical degradation, while still being slowly dissolved away. In this *in-vivo* trial, the physical properties of the soy-based CRD were shown to be sufficient to allow it to remain in the rumen and degrade slowly for 4 weeks, which is comparable to the results obtained by Waller *et al.* (2001a). The hydration-swelling of the CRD device inside the rumen is also a very important factor as it directly influences the rate of chlamydospore release. The CRD formulation used here demonstrated a slow rate of hydration over time and consequently its degradation rate was also slow, but with enough daily surface degradation to release an appropriate amount of viable chlamydospores.

The slow-release characteristics achieved and the viability of the released chlamydospores indicate that a soy-based CRD can be acceptable for future use as part of an integrated health plan to control nematodes in extensively grazing livestock. The future role of fungal CRDs in the control of parasitic nematodes of ruminants would be one of the prevention mechanisms rather than a cure. CRDs could be used at the time when pasture contamination with nematode eggs becomes a risk due to the consequent emergence of large quantities of infective larvae on pasture; thus the release of chlamydospores in faeces at that point would be effective in reducing the numbers of L3 migrating from faeces on to pasture (Waller *et al.*, 2001a). A practical means of administering the CRD in future applications could be through post-oral delivery using a bowling spear gun, whereby the CRDs would be placed directly into the rumen and so avoid the chewing process on initial ingestion.

The major consideration in the manufacture of any CRD containing nematophagous fungi is that the process used to form polymeric materials must not destroy the biological activity of the chlamydospores. High temperatures, the addition of chemical agents such as glutaraldehyde as cross-linking agents (Sagüés, 2012) and the use of high-pressure compression have commonly been used to fabricate polymers from soy protein, but may be damaging factors for the predatory ability of *D. flagrans*. In this experiment, the use of low temperatures and pressures and dialdehyde starch as a cross-linking agent proved to be innocuous. This is the first attempt to produce and test *in vivo* the performance of a CRD manufactured with biopolymer materials.

The numerous advantages of soy protein isolates as an economic, food-grade and biodegradable source of digestion-resistant polymers, and the capacity of fungal chlamydospores to withstand the passage through the gastrointestinal tract, are two important elements of the approach to nematode parasite control targeted here. Combination of these two elements shows great potential for creating a means of administration of fungal biological control agents with all the desirable characteristics of being environmentally innocuous, leaving no residues in animals, showing effective activity against anthelmintic-resistant nematodes, ease of applicability and inexpensive manufacture.

Acknowledgements

Archers Daniels Midland Co. (Decatur, Illinois, USA) is thanked for donating the SPI used in this study. Financial support from the Hannam Soy Utilization Fund and a Fund for Scientific and Technological Investigation (FONCyT) grant, PICT 33132, are gratefully acknowledged.

References

Araújo, J.V., Stephano, M.A. & Sampaio, W.M. (2000) Effects of temperature, mineral salt and passage through the gastrointestinal tract of calves on sodium alginate formulation of *Arthrobotrys robusta*, a nematode-trapping fungus. *Revista Brasilera de Parasitología Veterinaria* **9**, 55–59.

Caillard, R., Petit, A. & Subirade, M. (2009) Design and evaluation of succinylated soy protein tablets as delayed drug delivery systems. *International Journal of Biological Macromolecules* **45**, 414–420.

Casillas Aguilar, J.A., Mendoza de Gives, P., López-Arellano, M.E. & Liébano Hernández, E. (2008) Evaluation of multinutritional pellets containing *Duddingtonia flagrans* chlamydospore for the control of ovine haemonchosis. *Annals of the New York Academy of Sciences* **1149**, 161–163.

Cunningham, J.G. (1996) *Fisiología veterinaria*. Mexico, Editorial Interamericana, McGraw-Hill.

Githigia, S.M., Thamsborg, S.M., Larsen, M., Kyvsgaard, N.C. & Nansen, N.C. (1997) The preventive effect of the fungus *Duddingtonia flagrans* on trichostrongyle infections of lambs on pasture. *International Journal of Parasitology* **27**, 931–939.

Larsen, M., Faedo, M., Waller, P.J. & Hennessy, D.R. (1998) The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: studies with *Duddingtonia flagrans*. *Veterinary Parasitology* **76**, 121–128.

Maltais, A., Remondetto, G.F., Gonzalez, R. & Subirade, M. (2005) Formation of soy protein isolate cold-set gels: protein and salt effects. *Journal of Food Science* **70**, C67–C73.

Maltais, A., Remondetto, G.E. & Subirade, M. (2009) Soy protein cold-set hydrogels as controlled delivery devices for nutraceutical compounds. *Food Hydrocolloids* **23**, 1647–1653.

Maltais, A., Remondetto, G.F. & Subirade, M. (2010) Tabletted soy protein cold-set hydrogels as carriers of nutraceutical substances. *Food Hydrocolloids* **24**, 518–524.

Ribeiro Braga, F., Araújo, J.V., Silva, A.R., Araujo, J.M., Carvalho, R.O., Tavela, A.O., Campos, A.K. & Carvalho, G.R. (2009) Biological control of horse cyathostomin (Nematoda: Cyathostominae) using the nematophagous fungus *Duddingtonia flagrans* in tropical southeastern Brazil. *Veterinary Parasitology* **163**, 335–340.

Rosemberg, M. & Young, S.L. (1993) Whey proteins as microencapsulating agents. Microencapsulation of anhydrous milkfat-structure evaluation. *Food Structure* **12**, 31–41.

Sagüés, M.F. (2012) Control biológico de nematodos parásitos mediante hongos nematófagos: optimización de cultivos y su administración a través de polímeros de soja y bloques energéticos. PhD Thesis, Facultad de Ciencias Veterinarias. Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina.

Sagüés, M.F., Fusé, L.A., Fernández, A.S., Iglesias, L.E., Moreno, F.C. & Saumell, C.A. (2011) Efficacy of an energy block containing *Duddingtonia flagrans* in the control of gastrointestinal nematodes of sheep. *Parasitology Research* **109**, 707–713.

Sagüés, M.F., Saumell, C. & Fusé, L. (2012) *Control biológico de nematodos parásitos mediante hongos nematófagos*. Saarbrücken (Germany), Editorial Académica Española.

Song, F. & Zhang, L.M. (2009) Gelation modification of soy protein isolate by a naturally occurring cross linking agent and its potential biomedical application. *Industrial Engineering Chemistry Research* **48**, 7077–7083.

Song, F., Zhang, L.M., Yang, C. & Yan, L. (2009) Genipin-crosslinked casein hydrogels for controlled drug delivery. *International Journal of Pharmaceutics* **373**, 41–47.

Vaz, C.M., van Doeveren, P.F.M., Reis, R.L. & Cunha, A.M. (2003) Soy matrix drug delivery systems obtained by melt-processing techniques. *Biomacromolecules* **6**, 1520–1529.

Waller, P.J., Faedo, M. & Ellis, K. (2001a) The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: towards the development of a fungal controlled release device. *Veterinary Parasitology* **102**, 299–308.

Waller, P.J., Knox, M.R. & Faedo, M. (2001b) The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: feeding and block studies with *Duddingtonia flagrans*. *Veterinary Parasitology* **102**, 321–330.

Winzenburg, G., Schmidt, C., Fuchs, S. & Kissel, T. (2004) Biodegradable polymers and their potential use in parenteral veterinary drug delivery systems. *Advanced Drug Delivery Reviews* **56**, 1453–1466.