

A candidate gene marker for bloat susceptibility in cattle?

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ABSTRACT

Pasture bloat costs New Zealand's dairy industry at least \$25M annually. We have previously shown that a salivary protein is increased in abundance in saliva from cattle that have been selected for low susceptibility to bloat compared with those selected for high susceptibility. We have now obtained the cDNA sequence for two closely related forms of this protein, which we term bSP30a and bSP30b. These share 83% sequence identity at the amino acid level, but at best only 28-30% homology with salivary proteins from other species. Analysis of genomic DNA by PCR suggests that the two forms of bSP30 are coded on two separate genes. However, the function of bSP30 and how it might influence susceptibility to bloat remains as yet unknown.

Keywords: bSP30, saliva, protein, parotid, PSP

INTRODUCTION

Pasture bloat is a significant cost to the dairy industry. One recent estimate was that over \$25M is lost annually in animal deaths, labour costs and decreased production (Livestock Improvement Corporation unpublished data, 1997). Previous research has established that susceptibility to bloat varies considerably between animals, and is heritable (Morris *et al.*, 1997). Data from dairy cattle selectively bred for either low or high susceptibility to bloat over 8 generations best fit a model in which susceptibility is inherited as a single major recessive gene (Morris *et al.*, 1997).

Earlier physiological studies have shown differences in rumen volume, salivation rate, and salivary proteins between low and high susceptibility cattle (Carruthers *et al.*, 1988; McIntosh and Cockrem, 1977; Morris *et al.*, 1991). We have found that the abundance of a major salivary protein, which we have termed bovine salivary protein 30 kDa (bSP30) is increased on average by 66 ±15% in animals from the low susceptibility compared with the high susceptibility herd (Rajan *et al.*, 1996). Here we report the identification of two cDNAs coding for alternate forms of bSP30, and show that genomic DNA coding for both forms of bSP30 are found in all animals tested. In a preliminary analysis of two animals, we have also found that the abundance of bSP30 protein in the saliva reflects the abundance of bSP30 mRNA in the parotid gland from the same animal. These results suggest that variation in bSP30 abundance in saliva is the result of differences in bSP30 gene expression in the salivary glands.

MATERIALS AND METHODS

Cloning and sequencing of bSP30 cDNAs

A cDNA library was constructed in lambda phage using polyA RNA isolated from a bovine parotid salivary

gland. The reagents for construction of the library were obtained from Stratagene (La Jolla, CA) and the kit manufacturer's instructions were followed. The library was screened by filter hybridisation to a ³²P-labelled degenerate oligonucleotide (YTCBGGRTTRTTBGG) following standard procedures (Ausubel *et al.*, 1995). This sequence was based on a segment of bSP30 amino acid sequence (EPNNP) which was obtained by excising the bSP30 protein band from an acrylamide gel, digesting it with endoproteinase Lys-C, separating and recovering the proteolytic fragments by HPLC, and subjecting them to amino acid sequencing using a gas phase sequencer (model 470 A; Applied Biosystems, Foster City, CA) as previously described (Rajan *et al.*, 1996). Amino acid sequencing was performed by Catriona Knight, School of Biological Sciences, University of Auckland.

The cDNA inserts were sequenced using the double stranded method by the Waikato University automated DNA sequencer (Applied Biosystems model 377), and were found to contain open reading frames that were incomplete at the 5' end. Full length cDNAs for bSP30a and bSP30b were obtained by PCR of DNA isolated from the cDNA library using two nested 3' primers complementary to sequence within the bSP30 coding region and a 5' primer complementary to sequence within the cloning vector as described elsewhere (Haigh, 1995).

Detection of bSP30 genomic DNA

Genomic DNA (20 ng) was subjected to 30 cycles of PCR. For amplifying bSP30a, the mixture contained a common 5' primer and a specific bSP30a 3' primer. For amplifying bSP30b, a bSP30b-specific primer was substituted for the bSP30a-specific primer. Full details of the method are presented elsewhere (Haigh, 1995). The amplified DNA was analysed on a 1.8% (w/v) agarose gel and visualised by staining with ethidium bromide. Amplified

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DNA from some of the animals was digested with MspI and HinI. The amplified product from each of the primer pairs (70 µl) was divided into three portions; 30 µl for HinI digestion, 30 (l for MspI digestion, and 10 µl for undigested DNA. Digests were for 2 h at 37°C in the recommended buffer using 20 units of enzyme. Digests were subjected to electrophoresis in 8% (w/v) polyacrylamide gels containing 1 x TBE (90 mM Tris-Borate, 1 mM EDTA buffer, pH 8.0). The gel was then soaked for 5 min in 0.33 µg/ml ethidium bromide and the DNA visualised by UV fluorescence.

Gel analysis of salivary proteins

Saliva was collected from cattle as previously described (Rajan *et al.*, 1996) on the day before slaughter and assayed for protein by the Bradford method (Bradford, 1976) using reagent supplied by Bio-Rad (Hercules, CA). Saliva was lyophilised, resuspended in sample buffer to 1 mg/ml and subjected to electrophoresis on a discontinuous 12% (w/v) polyacrylamide-SDS gel and stained with Commassie blue using standard procedures (Ausubel *et al.*, 1995).

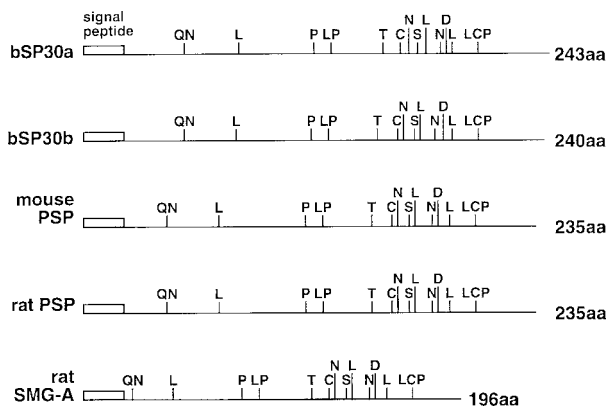
Northern blot analysis of parotid mRNA

Left and right parotid salivary glands were removed from cattle during abattoir processing, snap frozen in liquid nitrogen, and stored at -80°C. Parotid tissue was ground under liquid nitrogen, 0.2 g of the frozen powder was transferred to a 10 ml tube, 1.5 ml homogenising solution added (Trizol, Life Technologies, Auckland), and the freshly thawed tissue was homogenised using a mechanical homogeniser (Ultraturrax, Janke & Kunkel, Staufen, Germany). RNA was extracted from the homogenate following the Trizol manufacturer's procedure. The RNA was subjected to electrophoresis on formaldehyde/agarose gels, transferred to membrane, stained with methylene blue and photocopied, hybridised with ³²P-labelled bSP30 cDNA and exposed to film as previously described (Wheeler *et al.*, 1997).

RESULTS

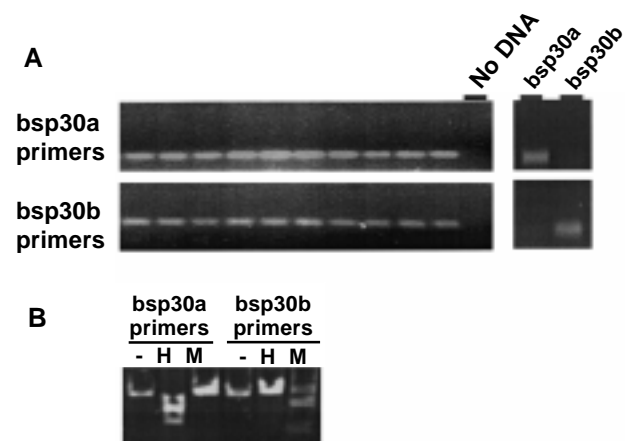
Two positive hybridising clones were isolated from the bovine parotid cDNA library and their insert DNA was sequenced. These proved to be incomplete at the 5' end. Subsequently, the full length contiguous sequence was obtained by PCR of the cDNA library. The resultant contiguous sequences each contained a single large open reading frame beginning with an ATG initiation codon (GenBank accession numbers U79413 and U79414). The predicted translation products, which we term bSP30a and bSP30b, contain 240 and 243 amino acids respectively and share 86% identity with each other. Both proteins also share 28-30% amino acid homology with Parotid Secretory Protein (PSP) and Submandibular Gland protein A from rodents. Despite this low overall homology, bSP30a and bSP30b share several structural features with these proteins, placing bSP30 in the PSP family of proteins (see Fig. 1). A full analysis of the bSP30a and bSP30b nucleotide and amino acid sequence has been presented elsewhere (Haigh, 1995).

FIGURE 1: Schematic of the primary structure of bSP30a, bSP30b and known members of the Parotid Secretory Protein family. The length of the solid lines indicate the total number of amino acids comprising the protein. The letters represent amino acids that are in common to all five proteins in a multiple sequence alignment performed using the GCG software package, and are placed according to their relative positions in the amino acid sequence.



The nucleotide sequences of bSP30a and bSP30b differ in 61 separate locations in their cDNAs, making it unlikely that they represent two alleles of the same gene. In order to confirm that bSP30a and bSP30b arise from two separate genes, we tested for their presence in genomic DNA from 18 cattle. A PCR product was obtained from all 18 cattle using both a bSP30a-specific and a bSP30b-specific primer set, strongly suggesting the existence of separate bSP30a and bSP30b genes. The results for ten cattle are shown in Fig. 2a. The bSP30a cDNA sequence contains a HinI restriction endonuclease cleavage site at nucleotide 82 while the bSP30b cDNA sequence contains an MspI site at nucleotide 70. Both sites are

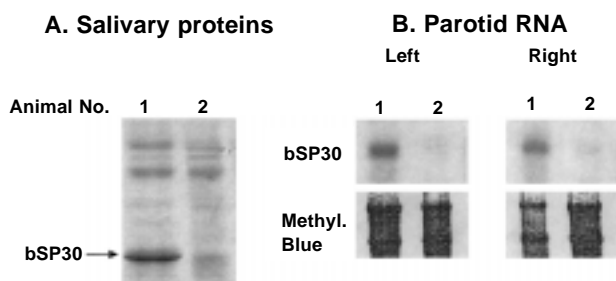
FIGURE 2: Detection of bsp30a and bsp30b sequences in genomic DNA. (A) Genomic DNA from 10 cattle was subjected to PCR using a bSP30a-specific (upper panel) and a bsp30b-specific primer set (lower panel) as described in the text. The PCR reaction mix loaded in lane 9 contained no template DNA. The PCR reaction mix loaded in the two lanes in the panel to the right contained bSP30a and bSP30b cDNA as template. (B) Genomic DNA from one animal was subjected to PCR as above, the amplified DNA was divided into portions which were not digested (-), digested with HinI (H) and digested with MspI (M). The DNA was then subjected to acrylamide gel electrophoresis.



within the segment that was amplified in Figure 2a. The amplified products from six animals were divided into portions and digested with *HinfI* and *MspI*. *HinfI* but not *MspI* digested the “bSP30a” products, while *MspI* but not *HinfI* digested the “bSP30b” products, confirming that both bSP30a and bSP30b sequences were amplified from each animal (Fig. 2b).

The variation between animals in bSP30 abundance in saliva may arise as a result of differences in the amount of bSP30 mRNA in the salivary glands. As a first step towards addressing this, the abundance of bSP30 mRNA was estimated in the left and right parotid salivary glands of two cattle that showed extremely high or low abundance of bSP30 in their saliva (Fig. 3a). The abundance of bSP30 mRNA was high in both the left and right parotid glands from the animal having a high abundance of salivary bSP30. Similarly, the bSP30 mRNA abundance was low in the animal having a low salivary bSP30 abundance (Fig3b).

FIGURE 3: Abundance of bSP30 in saliva and bSP30 mRNA in the parotid salivary gland. (A) Equal amounts (20 g) of saliva collected from two cattle were analysed by electrophoresis. (B) Equal amounts of parotid RNA (10 g) from the same animals as in Figure 3a were analysed by Northern blotting using a 900 base pair fragment of bSP30b cDNA as a probe. RNA from the left (two panels to the left) and right (two panels to the right) parotid glands from each animal were analysed separately. The blot was stained for RNA using methylene blue to visualise the 28S and 18S ribosomal RNA (lower panels) to confirm equal loading.



DISCUSSION

We have identified two closely related cDNAs, each coding for a different form of bSP30, and these appear to arise from separate genes. Based on sequence homology and conserved structural motifs, both forms of bSP30 belong to the Parotid Secretory family of proteins. None of these proteins have yet been ascribed a function. Elucidation of a role of bSP30 in conferring low susceptibility to bloat will therefore require experiments aimed at gaining a better understanding of the physical or biological properties of the protein.

Despite the small number of animals analysed, the Northern analysis results are consistent with the idea that the amount of bSP30 in the saliva is in large part deter-

mined by the amount of bSP30 mRNA present in the salivary glands. It follows then that the variation between animals could well be due to differences in the extent of bSP30 gene expression. It may therefore be pertinent to investigate the control of bSP30 gene expression in order to understand the underlying cause for the variation. These analyses did not distinguish between bSP30a and bSP30b. It will also be of interest to examine relative expression of these two mRNAs separately in a larger number of animals in order to test whether they are expressed independently or in concert.

Several aspects of bSP30 variability between cattle have not as yet been resolved. The amount of bSP30 mRNA in the parotid glands could reflect inherent genetic differences between animals, or alternatively that changes in abundance occur in response to systemic or environmental signals. It is conceivable that the level of bSP30 protein and mRNA could rise and fall over time. Also, we have not yet addressed whether the level of bSP30a or bSP30b mRNA is correlated with susceptibility to bloat, either in the Ruakura low and high susceptibility herds or in unrelated animals.

In conclusion, the data presented here establish the existence of two forms of bSP30 which appear to be coded on two separate genes. The abundance of bSP30 in the saliva may be due to the extent of bSP30 gene expression in salivary glands. However, the function of the two forms of bSP30 and their role in susceptibility to bloat is as yet unknown.

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