

PROTEIN REMOVAL FROM CATTLEHIDES DURING BRINE CURING I. IDENTIFICATION OF BOVINE SERUM ALBUMIN AS THE MAJOR SALT SOLUBLE PROTEIN COMPONENT

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Abstract

Fresh hides delivered within a few hours after slaughter from a packing house or brine cured hides preserved for many months can serve equally well as raw material for leather manufacture. Although leather with very similar characteristics can be produced from both, they do not respond identically to beamhouse processing. For example the penetration of chrome into different areas of the hide is more uniform in chrome tanned stock prepared from brine cured hides than from fresh hides. This difference may be due to the removal of proteins from the hide during the brine curing. Removal of extracellular protein may help to open up the hide structure allowing more rapid penetration of chemicals such as chrome during processing. This paper describes the extraction of proteins from cattlehides during brine curing. It demonstrates that the proteins removed from the hide vary qualitatively in different locations within the hide. A single protein, present throughout the hide, appears to constitute more than half of the total protein removed during curing. The purification and subsequent identification of that protein as bovine serum albumin (BSA) is reported.

Introduction

Tanning fresh hides creates both opportunities and problems for the tanner. Processing fresh hides significantly reduces salt pollution at both the tannery and the packing plant⁽¹⁾. However, because fresh hides must be processed within a few hours after slaughter, it is difficult to obtain a reliable supply of this raw material to maintain constant leather production. The compromise, in current use by a number of large tanneries, is to produce leather from a supply of fresh hides supplemented with brine cured raw material. Even when the source of supply is balanced, however, there are questions raised concerning quality differences in the leather manufactured from these two sources. One of the largest tanneries in this country has reported that when using identical formulae for processing fresh hides and brine cured hide there is a greater incidence of raw streaks in leather made from the fresh hides⁽²⁾. While there is no question that good quality leather can be produced from either, the conventional wisdom is that salt curing "changes the hide", in some presently undefined way, to prepare it for tanning. This investigation is directed toward understanding these changes by identifying the proteins removed from cattlehide during brine curing and soaking. This paper reports that a single protein was found to dominate the extracted proteins as observed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The purification and identification of this protein is described.

Experimental

PROTEIN EXTRACTION

Sections of fresh frozen cattlehide were used as starting material (20" x 20"). They were cut from a fleshed hide obtained from a local slaughter house within three hours of slaughter. Sections were placed in a drum containing a 400% brine float (4 gm saturated brine/gm hide) overnight. The drum was turned at four RPM for five minute intervals every four hours over a sixteen hour period. The hide samples were removed from the brine and the brine solution stored at 4°C. After soaking the samples in distilled water for sixteen hours the soak solution was separated from the hide samples and stored. All further work with these solutions was done at 4°C. The brine solution and the soak solution were each dialysed twice overnight against an 18:1 volume of distilled water to remove the salt. A precipitate, that formed during dialysis, was separated from the soluble fraction by centrifugation at 5000 times gravity for one hour in a Sorvall RC-5B centrifuge and discarded.

CORIUM PROTEIN EXTRACTION

A cattlehide is composed of two layers, the grain layer which contains the upper epidermis, vascular tissue, hair and sebaceous glands and the corium layer consisting almost entirely of collagen fibers. Corium tissue was prepared by removing the grain from frozen sections of hide with a mechanical splitter. Extraction of corium tissue and dialysis of the extract was carried out using the same procedures as outlined above for the whole cattlehide.

EXTRACTION OF CATTLEHIDE CROSS SECTIONS

One quarter inch diameter plugs were cut out of frozen cattlehides. The hair was removed with a razor. The plug was frozen and serially sectioned from grain to corium on a Lipshaw Model 275 CO₂ freezing microtome. The resulting sections were approximately 250 microns thick. Each section was weighed, placed in 40 µl of saturated sodium chloride solution and allowed to stand overnight at 4°C. One hundred µl of the extraction solution was removed and placed in a 2 ml eppendorf tube. The proteins were precipitated by adding 10 µl of saturated trichloroacetic acid to the extract. The tubes were held on ice for one hour and then centrifuged 10 minutes at top speed in a Beckman Microfuge Model E. The pellet was washed once with distilled water, centrifuged, and then dissolved in sample loading buffer (described under SDS-PAGE).

KJELDAHL NITROGEN

Kjeldahl nitrogen analysis was performed according to the ASTM standard method⁽³⁾.

PROTEIN DETERMINATION

Protein analysis was done using the Pierce protein analysis method⁽⁴⁾.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Qualitative analysis of the extracted proteins was performed by SDS-PAGE on a 12% gel crosslinked with 0.3% bis-acrylamide, containing 1% SDS⁽⁵⁾. The gels, 10 cm by 20 cm by 2 mm and containing 1% SDS, were run on a vertical electrophoresis apparatus (Model

100, Aquebogue Machine)^a. Five μ l of sample loading buffer (SLB)⁶ was added to twenty μ l of protein sample. Twenty μ l samples of this mixture were applied to the gel.

PROTEIN SILVER STAIN

Proteins were visualized in the SDS-PAGE gels with the protein silver stain procedure of Morrissey⁽⁷⁾.

BIO-GEL A CHROMATOGRAPHY

The dialysed supernatant from the corium extract was applied to a 2.5 cm x 25 cm column containing 100 to 200 mesh anion exchange gel DEAE Bio-Gel A at 0.5 ml/minute. The column was washed in 0.05 M phosphate buffer pH 7.0 until no further 280 nm absorbing materials eluted from the column. A 300 ml gradient of sodium chloride from 0 to 0.5 M was applied to the column at 0.5 ml/min and 5 ml fractions collected. A qualitative protein pattern was obtained for each fraction by SDS-PAGE.

PREPARATIVE ISOELECTROFOCUSING (IEF)

An LKB 2117 Multiphor II Electrophoresis System apparatus was used for preparative IEF. An Ampholine pH gradient from pH 4 to pH 7.0 was used as described in the LKB manual and originally reported by Radol⁽⁸⁾. Nineteen IEF fractions collected were individually desalted on Sephadex G-25 columns and analyzed by SDS-PAGE. The fractions ranged in pH from 3.40 to 6.9. Each fraction contained 2 to 3 mls. Several fractions that appeared to contain only one protein band were combined and lyophilized.

MOLECULAR WEIGHT DETERMINATION

Sedimentation velocity analysis of the purified protein was performed on a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and photometric scanner. Weight average molecular weights were obtained by fitting the data points by means of a nonlinear iterative Gauss-Newton regression analysis⁽⁹⁾.

SAMPLE PREPARATION FOR AMINO ACID ANALYSIS

Approximately 1 mg of lyophilized protein was hydrolyzed at 110°C in 1 ml of 5.7 N CH₁ containing (0.05%) phenol for 24 h in tubes evacuated to 20 mm and sealed. After hydrolysis the hydrochloric acid was removed on a rotary evaporator. The samples were syringe filtered before analysis⁽¹⁰⁾.

PERFORMIC ACID OXIDATION

The method of Hirs⁽¹¹⁾ as modified by Moore⁽¹²⁾ was employed for conversion of cystine to cysteic acid.

AMINO ACID ANALYSIS

Hydrolysate aliquots containing about 50 μ g of protein were analyzed on a Beckman

119CL amino acid analyzer interfaced with a Hewlett-Packard 3390A integrator. The standard protein hydrolysate procedure using a 0.6 x 22 cm column of W3H ion-exchange resin and a 96 min total analysis time was employed⁽¹³⁾.

WESTERN BLOT PROCEDURE

Lyophilized protein from the preparative IEF procedure was analyzed using the Western Blot procedure⁽¹⁴⁾. Samples of authentic BSA (Calbiochem 126609) and the purified protein were run side by side. Antibodies to BSA were purchased from Sigma Chemical Company.

Results and Discussion

The corium, or lower layer of the hide, is almost entirely made up of collagen fibers containing a small amount of vascular tissue. The collagen fibers contribute most of the physical strength of the skin. The grain, or surface layer of the hide, comprises about 10 to 15% of the skins thickness and contains most of the specialized tissues such as hair, hair follicles, muscles, sebaceous glands, and most of the capillary blood supply for the skin⁽¹⁵⁾. It was anticipated that more extractable nitrogen would be present in the grain layer, on a weight basis, than in the corium. Whole hide and corium samples were extracted with brine and then with water soak. After dialysis and centrifugation kjeldahl nitrogen determinations were done on both the supernatant and the pellet. The totals for these two fractions are presented in Table I. The pellet contained about 40 to 45% of the total nitrogen. About one half percent of the hide weight was removed during brine curing and soaking of the cattlehide samples. As expected more protein was removed from the whole hide extract than from the corium using the same procedure.

TABLE I

**Grams of Protein Removed From 1000 Grams of Cattlehide
During Brine Curing and Soaking***

	<u>Brine (gms)</u>	<u>Soak (gms)</u>	<u>Total (gms)</u>
Whole Hide	3.42	2.37	5.79
Corium	2.44	2.13	4.75

*Protein based on Kjeldahl nitrogen analysis

The protein extracts were examined qualitatively by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The whole hide extract appeared to contain a few more protein bands than the corium extract (Figure 1). There was no difference observed between the qualitative pattern of proteins in the brine and the soak solutions from either the whole hide or the corium. This suggests that the brine treatment does not extract all of the salt soluble proteins from the skin. More of the same proteins are extracted during the soak operations. The small amount of high molecular weight protein bands observed near the top of the gel indicated that very little native collagen is extracted by brine curing and soaking. There are a considerable number of protein bands present in the two extracts. However, electrophoresis of both the corium and the grain extracts (brine and soak) reveals one particular protein band that appears to be considerably higher in concentration than any of the others. Comparison of the electrophoretic patterns from corium tissue extracts to whole cattlehide tissue extracts also shows that the same protein band is dominant in both, but there is probably less additional protein present in the corium extract. Based solely on the intensity of the silver stain band it is possible that half

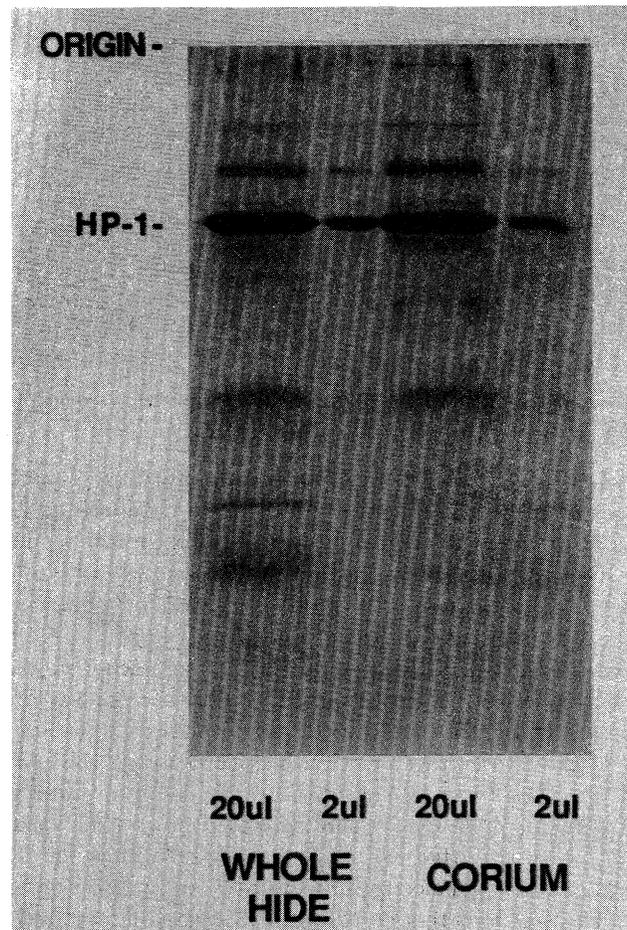


FIG. 1. — SDS-PAGE electrophoretic patterns of saturated brine extraction of whole corium and whole cattlehide samples. Each sample was run as extracted and at a ten fold dilution. The major protein band is Bovine Serum Albumin.

of the protein extracted from these hide tissues is a single protein. For ease of further discussion this protein will be referred to as HP-1 (hide protein 1).

To establish the location of HP-1 within the hide a quarter inch core of fresh frozen hide was removed and sectioned from grain to flesh as described in materials and methods. Sections were approximately 250 microns thick. Figure 2 is the SDS-PAGE pattern obtained from brine extracts of each individual section proceeding from the grain layer through to the flesh (right to left). The first sample is from the whole extract. The HP-1 protein band was found to be present throughout the hide from grain to flesh. This protein, as well as others observed on the SDS gels, are substantially removed during brine curing.

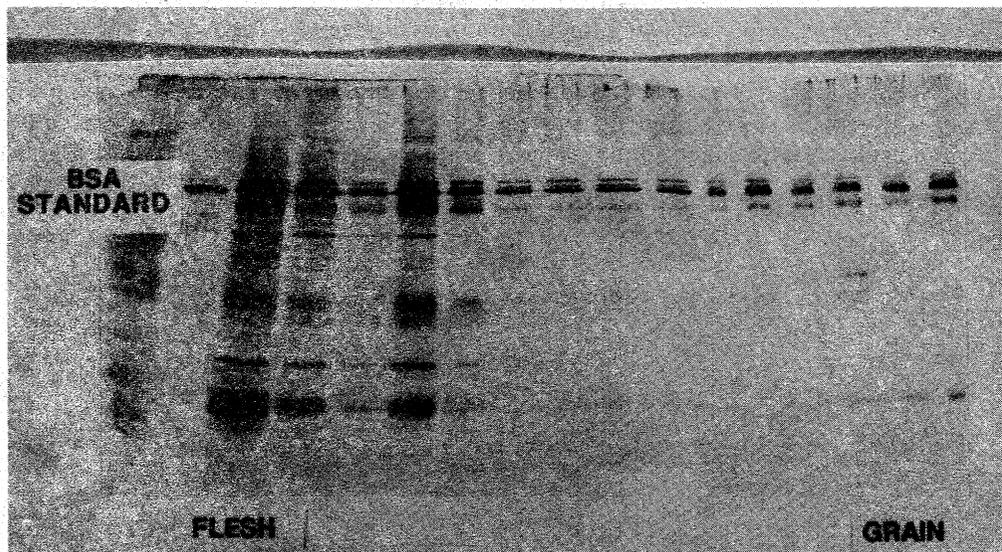


FIG. 2. — SDS-PAGE electrophoretic patterns from saturated brine extraction of sections of the hide proceeding from the flesh side of the hide to the grain layer.

Since these proteins largely remain in a fresh hide, even after soaking, differences in tanning properties in fresh hides compared to brine cured hides could be due to the presence of these proteins. In as much as half of the protein removed during brine curing appears to be a single protein, identification and characterization of this protein might provide insight into the basis for the observed differences in chrome penetration between fresh and brine cured hides.

Since the grain layer extract appeared to contain somewhat more protein relative to HP-1 when compared to the corium (Figure 1) purification was initiated with corium tissue. Corium brine extract was centrifuged and the supernatant dialysed twice overnight against distilled water. The extract was then passed through a DEAE Bio-Gel A ion exchange column. The HP-1 protein remained on the column. After washing the column with 0.05 M pH 7.0 phosphate buffer, 500 ml of a sodium chloride salt gradient from 0 to 0.5 M NaCl in the same buffer was applied to the column. Each fraction collected was analysed by SDS acrylamide gel electrophoresis to locate the fractions containing the HP-1 band (Figure 3). The fractions that contained the least contaminated proteins were combined, dialysed to remove salt, and lyophilized. These fractions contained several higher molecular weight bands that could be polymeric forms of BSA. They were not present in

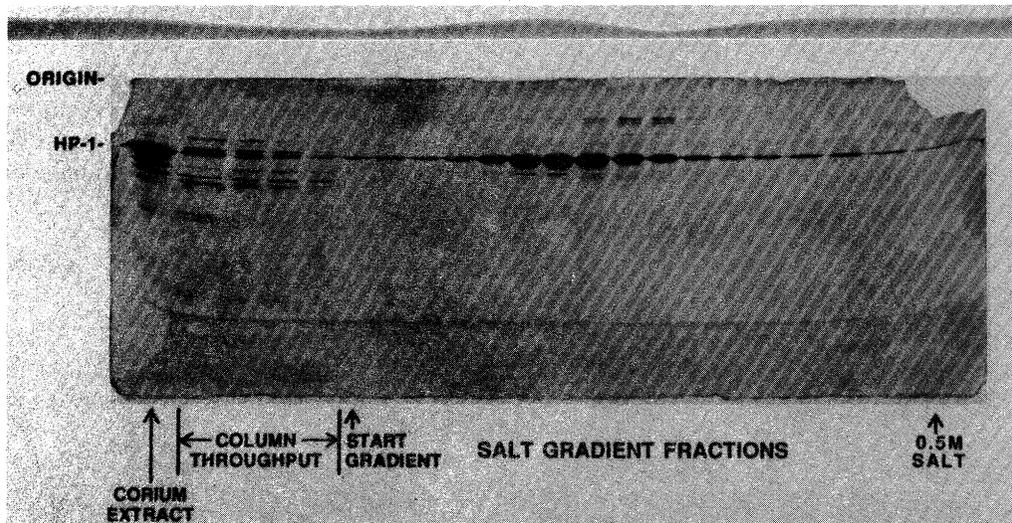


FIG. 3. — SDS-PAGE electrophoretic patterns from DEAE-Bio-Gel A column fractions from separation of proteins extracted by saturated brine from cattlehide corium.

the original extract and did not appear again. Their formation may have been related to the salt concentration in these fractions. The lyophilized material was redissolved in 0.05 M glycine and pH 3.5 to 10 ampholine solution and applied to an LKB preparative Isoelectric Focusing (IEF) apparatus. Figure 4 is the SDS PAGE gel of the 19 fractions. The HP-1 was found in fractions 16 to 19. The pI of the protein was pH 4.6 based on the pH of the fraction containing the largest quantity. The individual fractions near the isoelectric point varied in the amount of protein they contained but none appeared to contain contaminating proteins as determined by silver staining. The three most concentrated fractions were combined, dialysed overnight and lyophilized.

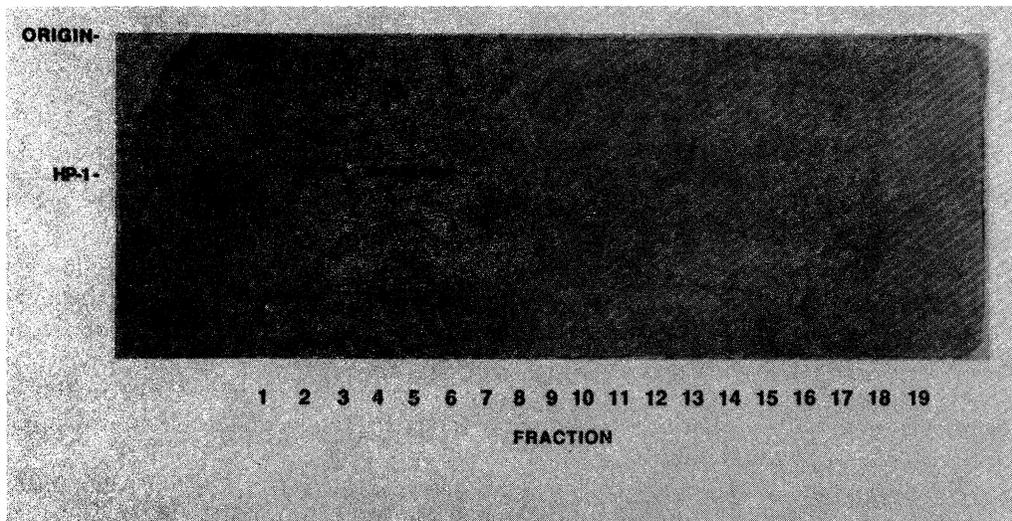


FIG. 4. — SDS-PAGE electrophoretic pattern of Preparative Isoelectric Focusing (IEF) fractionation of the purified major protein from DEAE-Bio-Gel-A column.

A sample of the purified protein, at a concentration of 0.5 mg/ml was run on a Beckman Model E analytical ultracentrifuge. The symmetrical shape of the first derivative of the concentration across the cell during the run suggested good homogeneity and based on the rate of sedimentation (Figure 5) an estimated molecular weight of 68,000. Calculation of the regression coefficient for sedimentation strongly suggested that the protein was homogeneous. The monomeric solution molecular weight observed in SDS indicates a lack

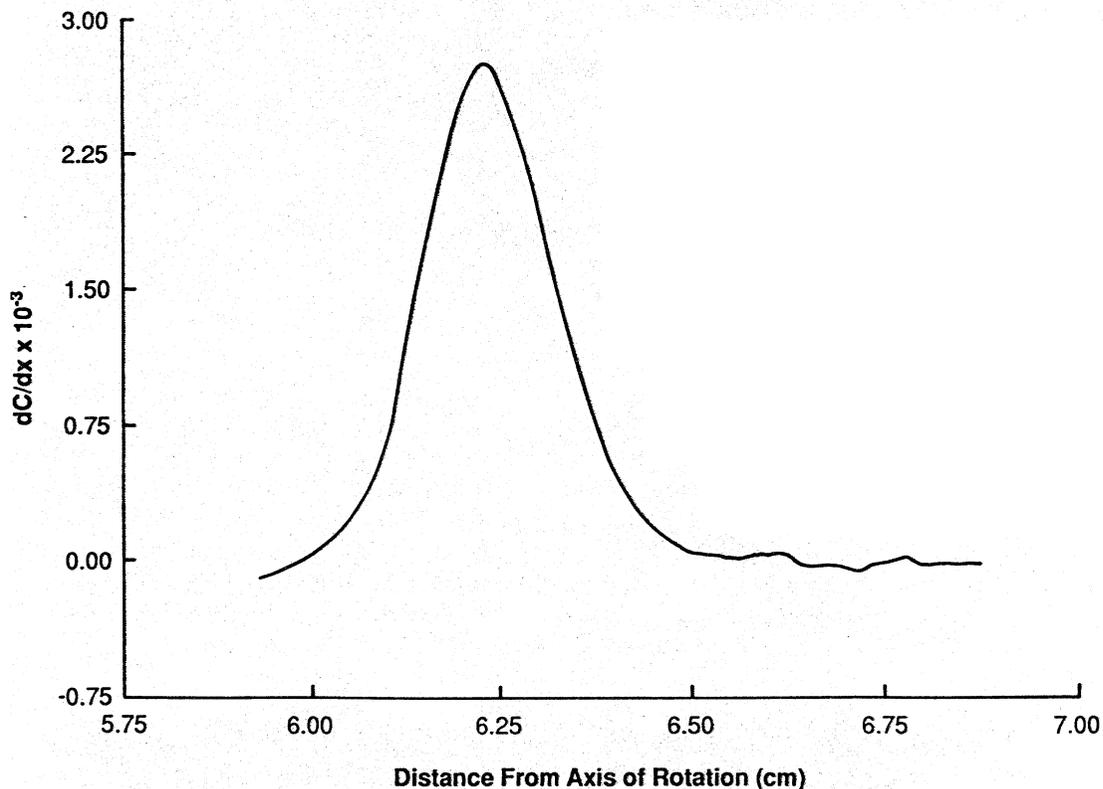


FIG. 5. — Analytical ultracentrifugation pattern during sedimentation of protein purified by preparative IEF. The photometric pattern displayed is derived from UV scans of an ultracentrifuge cell, fitted to the integral of a Gaussian and derivatized by a computer program. Analysis of peak position with time was used to estimate the molecular weight.

of aggregation or denaturation in the purified fractions. This information together with the results of the amino acid analysis of the protein (Table II), when compared with the known composition of Bovine Serum albumin (BSA)⁽¹⁶⁾, presented strong evidence that the isolated protein was BSA.

Further confirmation of the protein's identity was established by a Western Blot immunological analysis procedure which directly compared the purified protein with a standard sample of BSA. (Figure 6).

The next phase of this research will be to determine if Bovine Serum Albumin can affect the penetration of chrome into the hide if it is not removed prior to tanning. This may provide useful information to understand the difference between the tanning prop-

TABLE II

Amino Acid Composition - Molar Ratios

<u>Amino Acid</u>	<u>HP-1</u>	<u>BSA</u>
Lys	57.6	59.0
His	16.6	17.0
Arg	23.0	23.0
Asp	54.0	54.0
Thr	33.2	34.0
Ser	26.4	28.0
Glu	78.4	79.0
Pro	28.9	28.0
Gly	17.6	16.0
ALa	45.4	46.0
1/2 Cys (a)	37.0	35.0
Val	32.4	36.0
Met (a)	4.9	4.0
Ile	14.3	14.0
Leu	62.1	61.0
Try	20.4	19.0
Phe	26.1	27.0

a) Determined by performic acid oxidation

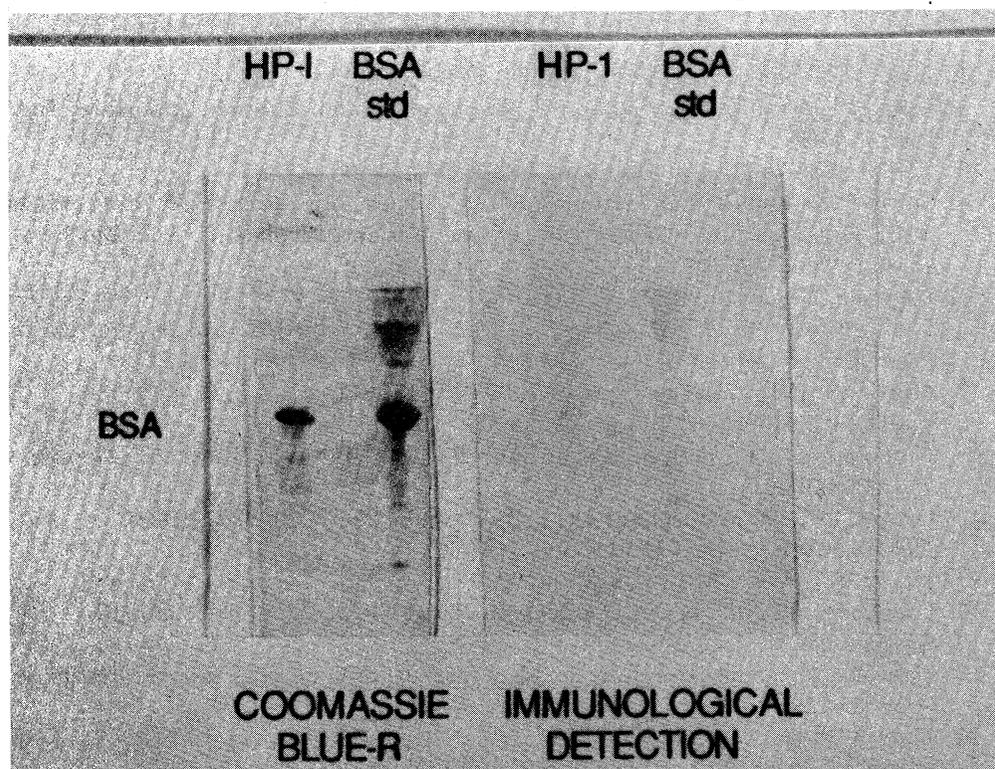


FIG. 6. — Western Blot comparison of purified corium protein and Bovine Serum Albumin standard.

erties of brine cured and fresh hides. The location of BSA throughout the skin, rather than just in the vicinity of the vascular system at the junction of the corium and the grain, suggests that there may be other roles for BSA in skin tissue other than as a carrier and buffer for the circulatory system, or that postmortum breakage of capillaries leads to very rapid diffusion of this protein throughout the hide.

Conclusions

Brine curing and soaking of cattlehides removes about 0.6% percent of the wet weight of the hide. Kjeldahl nitrogen analysis suggests that most of this material is protein. SDS acrylamide gel electrophoresis demonstrated that the majority of the protein removed was a single protein species distributed rather evenly across the hide samples. Purification and characterization of this protein revealed that the protein was bovine serum albumin. While BSA is a well known constituent of bovine serum, it has not been previously reported to be uniformly distributed throughout the hide. This finding suggests that there may be an additional role for BSA in the hide beyond its capacity to perform carrier and buffer roles in the circulatory system.

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