RESEARCHREPORT

PORK QUALITY

Title: Regulation of Fat Synthesis by ASP in the Pig – **NPB# 00-142**

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I. Abstract

Fat synthesis is regulated acylation stimulating protein (ASP) in human adipose tissue. To date, nothing is known about whether ASP has this function the pig, or even whether ASP exists the pig. We have now cloned a sequence of porcine nucleic acid that codes for ASP and verified that this protein exists in the pig. We have purified aproximately 10 mg of porcine ASP, developed a crude assay for porcine ASP, and verified that porcine ASP can promote fat synthesis in cultured human cells. We propose that ASP may play a role in regulation of energy storage in adipose tissue in the pig.

II. Introduction

Acylation Stimulating Protein (ASP) is believed to regulate lipid metabolism in adipocytes. In the search for causes of hyperapobetalipoproteinemia, Cianflone et al. (1) observed that adipocytes from afflicted patients were insensitive to a serum lipogenic factor. This factor, which they named ASP, can stimulate triacylglycerol synthesis in normal human adipocytes. Following purification, these researchers determined that ASP is identical to the complement C3 fragment, C3a, minus its C-terminal arginine (2). Purified human ASP (C3a-desArg) promotes fatty acid esterification, glucose uptake, and diacylglycerol acyltransferase activity in cultured human fibroblasts and adipocytes (3).

The pig's regulation of triacylglycerol synthesis is of considerable interest both as a model for the human and because excessive fat deposition can both increase production cost and reduce carcass value of agricultural swine. Therefore, we set out to develop methods for the study of ASP in the pig. We now report a method for purification of ASP from porcine serum and verification that porcine ASP can promote esterification of fatty acids.

III. Objectives

1. Purify 2 mg porcine acylation stimulating protein (ASP) from serum.
2. Test effect of ASP on fat synthesis in adipocytes.

IV. Procedures

Cloning of Porcine ASP

Total RNA was extracted from subcutaneous adipose tissue obtained at slaughter from male castrate pigs (body weight ~110 kg). A restricted set of porcine cDNA was synthesized from total RNA by reverse transcriptase (RT; Superscript II; Gibco BRL, Gaithersburg, MD) derived from two animals using a reverse primer, 5'-ACAGGAAGCTTGACAWGCTSACHGCCA-3' which flanks the 3' end of human ASP in the complement C3 gene. Three RT reactions were executed. Two reactions were independent but used RNA from a common pig. The third reaction used RNA from a second pig. A 228-bp ASP coding region was amplified from each set of cDNA by polymerase chain reaction (PCR) using the reverse primer above and a forward primer, 5'-GGYGTGTTYGTGCTGAAYAAGAA-3', which also flanks ASP in the human complement C3 gene (2,4). The PCR products were cloned into pBluescript 2KS+ (Invitrogen, Carlsbad, CA) and sequenced using T7 and T3 primers at the University of Nebraska DNA sequencing core facility with a Li-COR sequencer and dye primer chemistry (LI-COR, Lincoln, NE). The PCR conditions were: 1) denaturation 3 min at 94^{o}C; 2) one cycle of 15 sec at 94^{o}C, 2 min at 55^{o}C, and 2 min at 72^{o}C; 3) 37 cycles of 15 sec at 94^{o}C, 30 sec at 55^{o}C, and 30 sec at 72^{o}C; and 4) 7 min extension at 72^{o}C. All PCR reactions contained 1% DMSO.

ASP Western Blot Assay

We synthesized a peptide corresponding to the C-terminus of porcine ASP (CEYIAKLRQQHSRNKPLGLA) using FMOC-protected amino acids on a Pioneer peptide synthesizer (ABI, Inc.). The synthetic peptide was purified and conjugated to BSA essentially as described earlier (5) and used to immunize a rabbit. Antisera obtained after the fourth boost was used to probe western blots. Protein samples from 15% SDS-PAGE were transferred to nitrocellulose membranes by a semi-dry electric transfer apparatus (Semiphor, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Membranes were incubated with primary antibody raised against porcine serum

ASP polypeptide (1:500), or a primary antibody raised against human plasma ASP (1:1000; 6). Detection was by anti-rabbit IgG conjugated to alkaline phosphatase. The substrates were nitroblue tetrazoliun (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Purification of ASP

Fresh blood was obtained from white crossbred pigs at slaughter (body weight approximately 110 kg), allowed to clot 4 h at 4 ^{o}C, and centrifuged 20 min at 4,000 x g. Following the protocol for purification of human ASP (7), serum was spiked with inulin (0.5% w/v final concentration) and $MgCl_2$ (2 mM final concentration), and stirred 1 h at 37 ^{o}C to enhance activation of complement. Globular proteins were precipitated with concentrated HCl (1 M final concentration) and separated by centrifugation for 30 min at 4,000 x g. The supernatant was neutralized to pH 7.4 by addition of 10 N NaOH, stored at -20 ^{o}C and used for chromatographic purification of ASP.

The supernatant from the acid precipitation step was thawed on ice and applied to a C18 Sep-pak column (37 to 55 □m, 1.5 x 10 cm; Waters) previously wetted with 3 volumes methanol and equilibrated with 10 volumes 0.1% trifluoroacetic acid (TFA). Unbound proteins were removed by washing with 10 volumes of 20% acetonitrile (ACN)- 0.1% TFA. Bound proteins were eluted with 6 volumes of 80% ACN- 0.1% TFA. One column volume fractions were collected. Fractions rich in ASP based on 15% SDS-PAGE and immunoblotting analysis (generally fractions one to two) were pooled, lyophilized, and redissolved in buffer A (10 mM Tris, 10 mM NaCl, pH 7.1) plus 0.1% TFA. The dissolved sample was further fractionated by cation exchange (Poros PE-Biosystems, HS/M, 4.6/100 mm, 1.662 ml) pre-equilibrated with 10 volumes buffer A using an ABI BioCAD Workstation. After washing with five column volumes of buffer A, bound proteins were eluted with a linear 0 to 1.5 M gradient of NaCl in buffer A at a flow rate of 4 ml/min over ten column volumes. The elution peak (corresponding to 1 M NaCl) was pooled and directly loaded into a reversed-phase column (Poros PE-Biosystems, R1/H, 4.6 x 100 mm, 1.662 ml) pre-equilibrated with 15% ACN/0.1% TFA using an ABI BioCAD workstation. After washing with three column volumes of 15% ACN/0.1%TFA, bound proteins were eluted with a linear gradient from 15% ACN/0.1% TFA to 85% ACN/0.1%TFA at a flow rate of 3 ml/min over 15 column volumes. Peaks were pooled from several runs and lyophilized. Purified porcine ASP was reconstituted and dialyzed into 10 mM sodium phosphate buffer, pH 7.1, (4 ^{o}C) and stored frozen (-80 ^{o}C). Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL) using bovine albumin as a standard.

Mass Spectroscopy

Electrospray mass spectroscopy was performed on purified ASP obtained after reverse-phase chromatography at the University of Nebraska mass spectroscopy core facility using a Finnigan LCQ ion trap mass spectrometer.

Amino Acid Sequencing

Approximately 3 □g of purified ASP protein was fractionated by 15% SDS-PAGE, and electroblotted to a polyvinylidene difluoride membrane. The membrane was stained with amido black (8) and bands corresponding to ASP were excised and sequenced on an ABI-Procise 494 sequencer using manufacturer-suggested protocols for 20 cycles.

Acylation Stimulating Activity Assay

Human skin fibroblasts were plated in Dulbecco's modified eagle medium/F12 containing 100 □M [9,10-^{3}H(N)]-oleic acid:BSA (5:1) and grown to 90% confluence according to Baldo et al. (7). Cultures were treated for 6 h with: PBS (n = 9); 5 □M human ASP (n = 9); or 5 □M porcine ASP (n = 3). An organic extract of each culture was subjected to thin layer chromatography and triacylglycerol fractions were analyzed in a □-counter to quantify oleate esterification.

V. Results

cDNA Sequence

The RT-PCR 580-base-pair-product was completely homologous to porcine complement C3 (Accession #110278). This sequence exactly matches a subsequent full length clone (Accession #154933). The region encoding ASP is aligned with human ASP in Fig. 1. This region possibly has two polymorphisms which do not impact amino acid sequence. Base 187 was C versus T and base 207 was C versus A in one of two independent clones sequenced. Porcine ASP nucleic acid sequence is 79.4% homologous to human (4; K02765), 72.4% homologous to mouse (9; K02782), 74.6% homologous to rat (9; X52477), and 86.0% homologous to bovine (Accession #AF110279). The porcine cDNA predicts a 76-amino acid ASP sequence which is 75.0, 78.9, 76.3, and 84.4% similar to human, mouse, rat, and bovine ASP amino acid sequences, respectively.

ASP Purification

Porcine ASP was obtained from serum in four steps. First, complement was activated with inulin, and extraneous proteins were acid-precipitated. The remaining supernatant was subjected sequentially to C-18 chromatography and cation exchange chromatography. A major peak was eluted by 1 M NaCl in the cation exchange column and collected as ASP-rich fractions (Fig. $2A_1$) based on SDS-PAGE (Fig. $2A_2$) and western blot analyses (Fig. $2A_3$). Following a second reverse-phase step in which ASP eluted at approximately 43% ACN as a single, dominant peak (Fig. 2B), the resulting porcine ASP was near homogeneity (Fig. 3, lane 5). The yield from 400 ml of porcine serum was 5.5 mg of ASP. The relative purification and cumulative ASP yield at each step are presented in Table 1 and Fig. 3.

ASP Characterization

The molecular mass of purified porcine ASP determined by mass spectrometry was 8,926 which corresponds to the calculated mass of the predicted amino acid sequence. Porcine ASP migrates slightly faster in 15% SDS-PAGE (estimated 8.9 kDa; Fig. 4), than human ASP, perhaps because the human protein is slightly larger (MW = 8,932). N-terminal sequencing of 20 residues of porcine ASP revealed an exact match to the predicted sequence (Fig. 1).

Our antisera raised against porcine ASP polypeptide does not cross-react with human ASP (Fig. 4) although it clearly can detect porcine ASP on Western blots. Similarly, the antibody raised against purified human ASP (6) does not react strongly with porcine ASP on Western blots. Assay of ASP in porcine serum can be made, however, if the serum is acid precipitated. We observed that the concentration of ASP was elevated in pigs following a high fat meal vs following an overnight fast (not shown). This compares favorably to the response of ASP to fat consumption in humans (10).

Porcine ASP stimulated esterification of oleate into triacylglycerol in human fibroblasts. Mean (+/- SD) esterification was 108 (+/- 8.5), 218 (+/- 16.6), and 217 +/-

41.9 pmol triacylglycerol/mg cell protein for control, 5 □M human ASP, and 5 □M porcine ASP treatments, respectively.

In conclusion, this communication indicates that ASP can be obtained from pig blood and that porcine ASP exhibits activity comparable to ASP derived from human blood. Like human ASP, it is resistant to treatment with strong acid and hydrophobic treatments. This information should be of value to those studying regulation of adiposity in swine, given the dearth of hormones known to promote triacylglycerol synthesis in porcine adipose tissue. We propose that ASP may play a role in regulation of adipose tissue function in the pig.

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TABLE 1
ASP Purification from Porcine Serum

Purification Step	Protein[a] (mg)	ASP[b] (mg)
Serum (400 ml)	28,000	ND[c]
Neutralized acid supernatant (246 ml)	3,100	28
C-18	30.6	8.0
HS/M	10.2	6.9
R1H	5.5	5.5

[a]Total protein concentration was determined by the BCA procedure except that protein content of serum was assumed (11). [b]ASP protein was quantified by scanning Coomassie blue-stained SDS-PAGE gels using a Hitachi Genetic Systems instrument (GeneSnap 2.60.0.14; Synoptics Ltd.; Cambridge, England). For the above calculations, the R1H-eluted ASP was considered pure and thus used as standard for quantifying ASP in the other samples (CCDBIO GeneTools Version 2.12.05; Hitachi Genetic Systems; Alameda, CA). [c]Not determined.

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FIG. 1. Region of porcine complement C3 cDNA which encodes ASP, predicted ASP protein sequence, and alignment with human complement C3 (4). Analysis of two independent clones revealed a possible polymorphism at positions 187 (C vs. T), and 207 (C vs. A) which does not affect predicted amino acid sequence. The above sequence exactly matches that reported by Wimmers et al. (12).

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Pig      S   V   Q   L   M   E   K   R   M   D   K   L   G   Q   Y   S     16
Human    S   V   Q   L   T   E   K   R   M   D   K   V   G   K   Y   P
Pig      tcc gtg cag ctc atg gag aaa agg atg gac aaa ctg ggt cag tac agc   48
Human    tcc gtg cag ctc acg gag aag cga atg gac aaa gtc ggc aag tac ccc

Pig      K   D   V   R   R   C   C   E   H   G   M   R   D   N   P   M     32
Human    K   E   L   R   K   C   C   E   D   G   M   R   E   N   P   M
Pig      aag gac gtg cgc aga tgc tgt gag cat ggc atg cgg gac aac ccc atg   96
Human    aag gag ctg cgc aag tgc tgc gag gac ggc atg cgg gag aac ccc atg

Pig      K   F   S   C   Q   R   R   A   Q   F   I   Q   H   G   D   A     48
Human    R   F   S   C   Q   R   R   T   R   F   I   S   L   G   E   A
Pig      aag ttc tcg tgc cag cgc cgg gct cag ttc atc cag cat ggt gat gcc  144
Human    agg ttc tcg tgc cag cgc cgg acc cgt ttc atc tcc ctg ggc gag gcg

Pig      C   V   K   A   F   L   D   C   C   E   Y   I   A   K   L   R     64
Human    C   K   K   V   F   L   D   C   C   N   Y   I   T   E   L   R
Pig      tgc gtg aag gcc ttc ctg gac tgc tgc gaa tac atc gca aag ttg cgg  192
Human    tgc aag aag gtc ttc ctg gac tgc tgc aac tac atc aca gag ctg cgg

Pig      Q   Q   H   S   R   N   K   P   L   G   L   A                     76
Human    R   Q   H   A   R   A   S   H   L   G   L   A
Pig      cag cag cac agc cga aac aag ccc ctg ggg ctg gcc                   228
Human    cgg cag cac gcg cgg gcc agc cac ctg ggc ctg gcc
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FIG. 2. Purification of ASP from porcine serum. **A_1.** After primary reverse phase C18 chromatography, ASP- containing fractions were eluted from an HS/M cation exchange column with a linear 0-1.5 M gradient of NaCl (dashed line) and pooled based on SDS-PAGE and Western analyses. Porcine ASP eluted at 1 M NaCl. **A_2** The Coomassie brilliant blue-stained 15% SDS-PAGE of selected fractions referenced to fraction numbers from panel A_1. Each sample was concentrated, desalted and dissolved in Lammeli buffer before electrophoresis. **A_3** Western blot of samples identical to those in panel A_2 probed with anti-porcine ASP polypeptide antibody. **B.** R1 H reverse - phase chromatography of ASP-containing fractions from HS / M column. Proteins were eluted with a linear 15% to 85% ACN gradient (dashed line). A sharp, single peak rich in ASP was pooled and lyophilized.

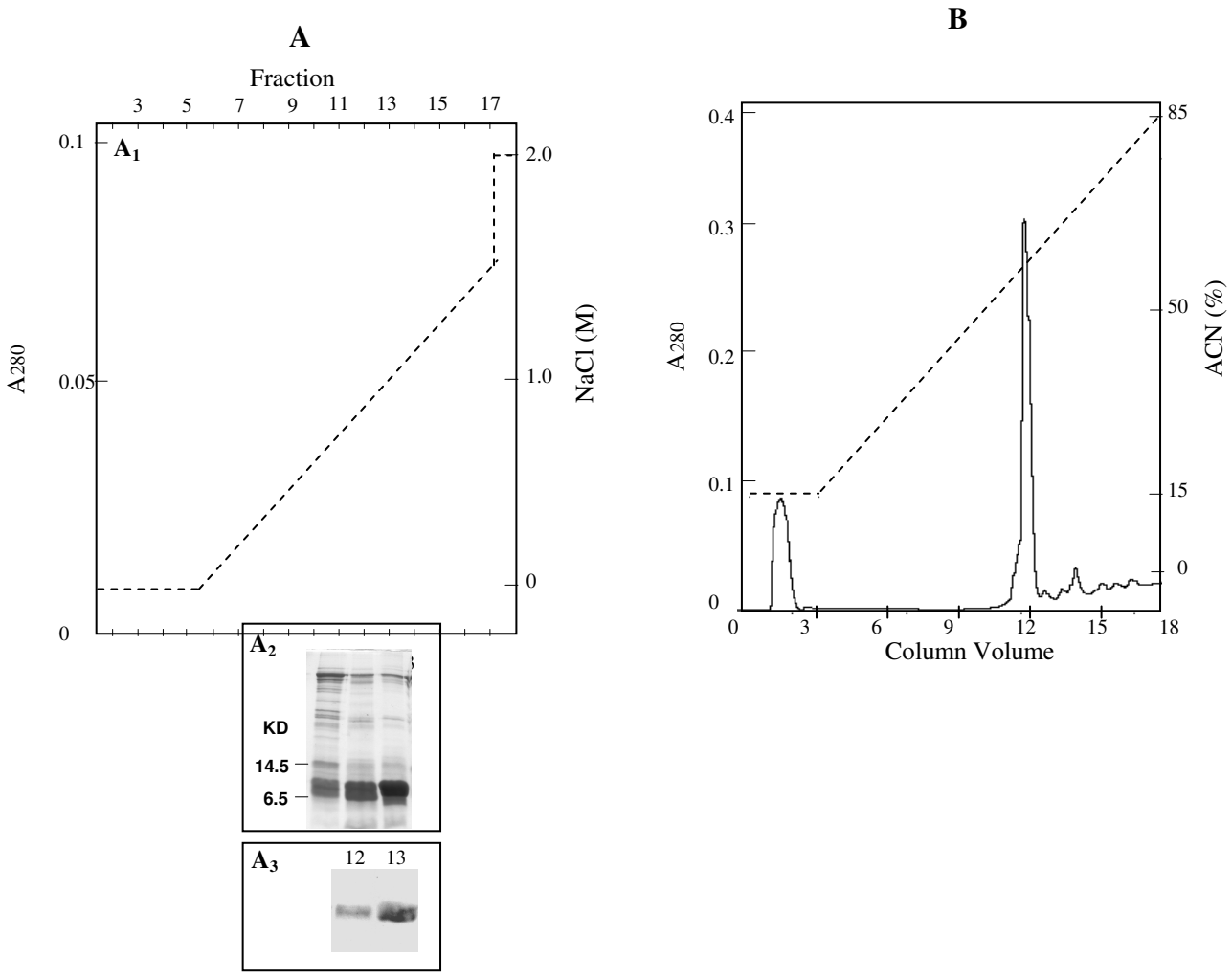

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FIG. 3. SDS-PAGE (15%) analysis of chromatographic steps in the purification of ASP from porcine serum. **Lanes 1 and 6:** Bio-Rad broad range marker. **Lane 2:** Clarified supernatant after acid precipitation and neutralization. **Lane 3** Fractions pooled from C18 reverse – phase. **Lane 4:** Fractions pooled from HS/M cation exchange. **Lane 5:** Fractions pooled from R1H reverse – phase.

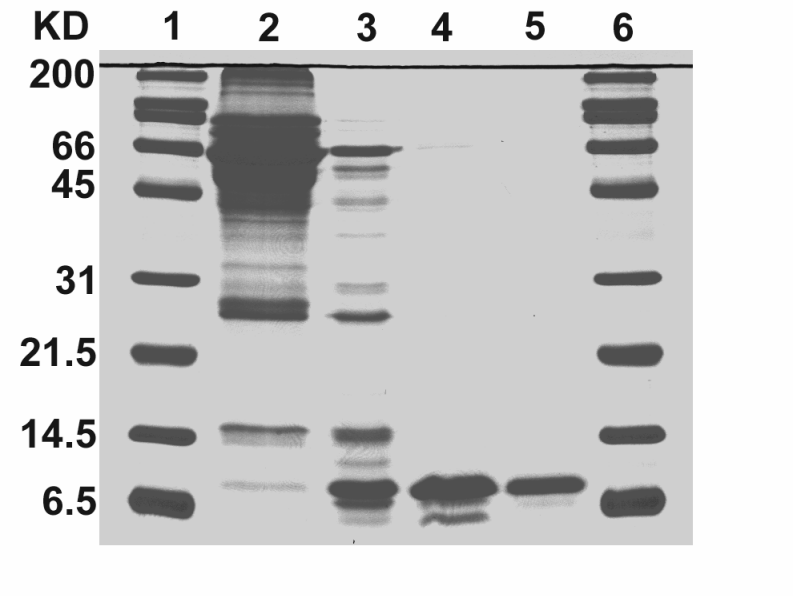

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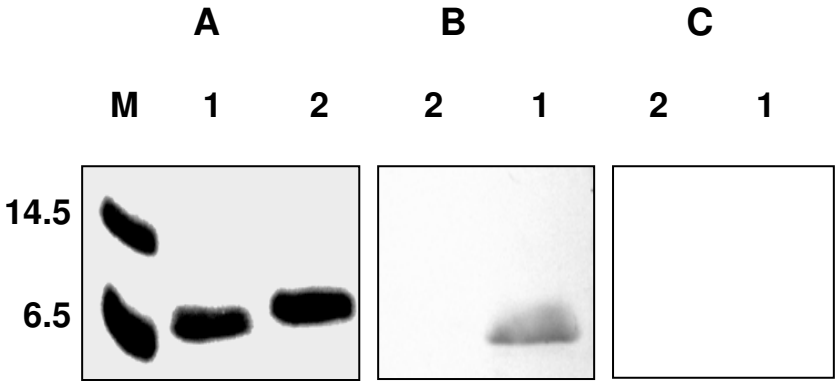
FIG. 4. SDS-PAGE and Western analyses of purified porcine and human ASP. **A.** SDS-PAGE analysis. Lane 1: Porcine ASP (2ug); Lane 2: Human ASP (2ug); Lane M: Molecular weight markers (kDa). **B.** Western blot of the same samples as in panel A probed with anti-porcine ASP polypeptide antibody. **C.** Western blot of the same samples as in panel A probed with anti-human ASP antibody.



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