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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.

**Materials and methods**

**Cloning of porcine ASP**

Total RNA was extracted from subcutaneous adipose tissue obtained at slaughter from male castrate pigs (Yorkshire/Landrace/Duroc crossbred, 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

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**Purification and characterization of acylation stimulating protein from porcine serum**

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**Abstract**

A method for purifying acylation stimulating protein (ASP) from porcine serum is described. The mRNA encoding ASP was cloned by reverse transcriptase-polymerase chain reaction which predicted a 76 residue peptide. Based on this sequence, we generated antisera to a C-terminal peptide (ASP\(_{1-20}\)) which aided ASP purification. Identity of the purified protein was verified by N-terminal sequencing. The molecular mass of porcine ASP is 8926. Porcine ASP stimulated esterification of fatty acid into triacylglycerol in cultured human cells with potency similar to that of human ASP (twofold at 5 μM). Based on this evidence that ASP exists in porcine blood, and that it has acylation stimulating activity, we propose that ASP may play a role in regulation of energy storage in adipose tissue in the pig.

**Keywords:** Pig, Complement factor C3a, Acylation stimulating protein, Adipose tissue

**Abbreviations:** ASP, acylation stimulating protein; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline solution; PCR, polymerase chain reaction; RT, reverse transcriptase; TFA, trifluoroacetic acid.
Purification of ASP

We synthesized a peptide corresponding to the C-terminus of porcine ASP (CEYIAKLRQHSRNKPLGLA) using FMOC-protected amino acids on a Pioneer Peptide Synthesizer (ABI). 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, Gaithersburg, MD). Membranes were incubated with primary antibody 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 tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Amino acid sequencing

Approximately 3 μg 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’s medium/F12 containing 100 μM [9,10-3H(N)]oleic acid:BSA (5:1) and grown to 90% confluency according to Baldo et al. [7]. Cultures were treated for 6 h with PBS (n=9); 5 μM human ASP (n=9); or 5 μM 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′-GGYGTTGTTYGCTGAAAYAAGAA-3′, which also flanks ASP in the human complement C3 gene [2 and 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 °C; (2) one cycle of 15 s at 94 °C, 2 min at 55 °C, and 2 min at 72 °C; (3) 37 cycles of 15 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and (4) 7 min extension at 72 °C. All PCRs contained 1% DMSO.

ASP Western blot assay

We synthesized a peptide corresponding to the C-terminus of porcine ASP (CEYIAKLRQHSRNKPLGLA) using FMOC-protected amino acids on a Pioneer Peptide Synthesizer (ABI). 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, Gaithersburg, MD). Membranes were incubated with primary antibody 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 tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

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.

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porcine ASP (n=3). An organic extract of each culture was subjected to thin layer chromatography and triacylglycerol fractions were analyzed in a β-counter to quantify oleate esterification.

Results and discussion

cDNA sequence

The RT-PCR 580-bp product was completely homologous to porcine complement C3 (Accession No. 110278). This sequence exactly matches a subsequent full-length clone (Accession No. 154933). The region encoding ASP is aligned with human ASP in Figure 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 the 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 No. 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 C18 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 (Figure 2A) based on SDS–PAGE (Figure 2A) and Western blot analyses (Figure 2A). Following a second reverse-phase step in which ASP eluted at approximately 43% ACN as a single, dominant peak (Figure 2B), the resulting porcine ASP was near homogeneity (Figure 3, lane 5). The yield from 400 ml porcine serum was 5.5 mg ASP. The relative purification and cumulative ASP yield at each step are presented in Table 1 and Figure 3.

ASP characterization

The molecular mass of purified porcine ASP determined by mass spectrometry is 8926 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; Figure 4A, lane 1, 2) than human ASP, perhaps because human ASP is slightly larger (MW=8932). N-terminal sequencing of 20 residues of porcine ASP revealed an exact match to the predicted sequence (Figure 1).

Our antisera raised against porcine ASP polypeptide do not crossreact with human ASP (Figure 4B, lane 2), although it can clearly detect porcine ASP on Western blots (Figure 4B, lane 1). Similarly, the antibody raised against purified human ASP [6] does not react strongly with porcine ASP on Western blots (Figure 4C, lane 1). 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 versus 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 μM human ASP, and 5 μM porcine ASP treatments, respectively. We have not investigated the mechanism by which porcine ASP enhances fatty acid esterification. Presumably it functions in the same manner as human ASP. Human ASP stimulates diacylglycerol acyltransferase, apparently via protein kinase C signalling from a cell surface receptor [13]. The effect of ASP on incorporation of fatty acid into triacylglycerol can be attributed to esterification. Van Harmelen et al. [14] have reported that, like insulin, ASP increases esterification, but unlike insulin, ASP does not influence lipolysis. Given the significant concurrent cycling of esterification and lipolysis, a small increase in esterification will impart a large increase in net triacylglycerol accumulation, assuming a constant rate of lipolysis. Consequently, the twofold enhancement of esterification caused by porcine ASP is evidence that this molecule may be a powerful determinant of adipose tissue lipid metabolism. Interestingly, we observed only a modest effect of porcine ASP on lipid syn-
thesis in porcine adipose tissue explant cultures [15]. However, this difference in response as compared to the cell cultures reported here may be due to technical problems of making such measurements in tissue pieces versus monolayers of cells. On the other hand, it remains to be demonstrated that porcine adipocytes are responsive to porcine ASP.

We conclude 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 the complement proteins, ASP and adipsin [16], may play a role in regulation of adipose tissue function in the pig.

Table 1. ASP purification from porcine serum

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein(^a) (mg)</th>
<th>ASP(^b) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (400 ml)</td>
<td>28,000</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>Neutralized acid supernatant (246 ml)</td>
<td>3100</td>
<td>28</td>
</tr>
<tr>
<td>C18</td>
<td>30.6</td>
<td>8.0</td>
</tr>
<tr>
<td>HS/M</td>
<td>10.2</td>
<td>6.9</td>
</tr>
<tr>
<td>R1H</td>
<td>5.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

\(^a\) 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 (Gene-Snap 2.60.0.14; Synoptics; 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 Gene-Tools Version 2.12.05; Hitachi Genetic Systems; Alameda, CA).

\(^c\) Not determined.
Acknowledgments

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References


Figure 4. SDS–PAGE and Western analyses of purified porcine and human ASP. (A) SDS–PAGE analysis. Lane 1: Porcine ASP (2 μg); Lane 2: Human ASP (2 μg); 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.